An antibiotic triggers cell stress responses and Epstein Barr virus lytic activation

by

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ABSTRACT

Epstein–Barr virus (EBV) is a human gammaherpesvirus, which infects most of the human population worldwide, and is one of the first human viruses found to be associated with cancer. EBV has a latent lifecycle characterized by limited gene expression, and a lytic lifecycle where infectious virions are produced. Latent infection is responsible for causing various types of cancers, and inducing the EBV lytic cycle has been suggested as a new approach for treating these types of malignancies. The unfolded protein response (UPR) is one of the major pathways which leads EBV to enter the lytic replication cycle. Thus several UPR inducers such as bortezomib are widely used for activating EBV in various cancer cell lines in laboratory. Recent investigations have demonstrated that clofoctol, an antibiotic drug that has anti-proliferative activities in prostate cancer cell lines, activates the UPR. In order to investigate whether clofoctol activates the UPR and leads to the viral activation, we tested the drug on various types of EBV-positive cancer cell lines, including the BX1-Akata Burkitt lymphoma cell line, lymphoblastoid cell line (LCL), SNU719 gastric cancer cell line and C666 nasopharyngeal cancer cell line.

Here, we show that clofoctol induces EBV lytic activation in a variety of cancer cell lines at a clinically achievable concentration. The effect was similar to that seen with bortezomib, but was more rapid and worked in broader range of the cell lines. Viral immediate early lytic gene Zta, early lytic gene Bmrf1 and late lytic gene gp350 expression were increased in RNA level. Increased Zta protein and EBV DNA copy number were confirmed in BX1-Akata cells. However, the expression of late protein gp350 was not detected after clofoctol treatment. A Raji infection assay using EBV

expressing GFP showed that clofoctol treatment does not lead to infectious virion production.

Similar to a previous report with prostate cancer cell lines, clofoctol induces all three pathways of UPR – PERK, IRE1 and ATF6. Activation of the PERK branch was confirmed by demonstration of increased eIF2α phosphorylation, and increased ATF4 and CHOP expression. Activation of IRE1 and ATF6 pathways was demonstrated by an increase in XBP1 spliced RNA and Bip RNA, respectively. With modulation of the PERK branch by shRNA-mediated knockdown and a PERK-specific inhibitor, we found that the PERK pathway mediates EBV lytic activation by clofoctol as inhibited PERK led to reduced Zta expression. Activated PERK leads to eIF2α phosphorylation which in turn inhibits most translation, but allows some RNAs including ATF4 to be translated. In our results, Zta protein synthesis was enhanced with PERK activation and eIF2α phosphorylation by clofoctol treatment, suggesting Zta translation overcomes the block to translation mediated by eIF2α phosphorylation. Furthermore, we found the PERK pathway differentially regulates viral lytic gene expression at the protein level. Clofoctol treatment alone triggers expression of the immediate early protein Zta. However, ZTA expression was reduced by treatment with a PERK inhibitor. In contrast, expression of the viral late lytic protein gp350 was not increased by clofoctol alone but was increased by the combination of clofoctol and PERK inhibitor. This result suggests that clofoctol activates other branches of the UPR and that these also are important in driving EBV lytic infection.

eIF2α phosphorylation by PERK is also a part of the integrated stress response (ISR) and there are more kinases that induce the phosphorylation of eIF2α, including

HRI, PKR, and GCN2. In order to demonstrate that activation of all eIF2α kinases results in EBV lytic induction, we performed pharmacologic inductions of four kinases and evaluated the effect on virus in BX1-Akata cells. The specific activation of each of the eIF2α kinases triggered EBV Zta RNA expression and increased GFP expression in BX1-Akata cells.

Our results show that clofoctol activates EBV lytic gene expression, and that this expression is partially mediated by the PERK pathway of UPR and ISR. Inhibition of global protein translation by the PERK pathway allows immediate early Zta protein synthesis but blocks late protein gp350 synthesis and virion production. We also found that other ISR stimuli can lead to viral lytic induction. These results suggest the possibility of new approaches to modulate viral gene expression for therapeutic purposes as well as providing new insights into physiologic stimuli that may trigger EBV reactivation *in vivo*.

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Finally, I dedicate this paper to my beloved family. I am the luckiest girl on earth to have you as my parents and big brother. Thank you, grandmothers for constantly praying for me, and thank you everyone else in my family for your endless support and love. I will commit myself to sharing this love with the world.

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ABBREVIATIONS

AIDS	Acquired immunodeficiency syndrome
ATF	Activating transcription factor
Anti-IgG	Anti-Immunoglobulin G
BIP	Binding immunoglobulin protein
BZ	Bortezomib
CEBPβ	CCAAT/enhancer-binding protein beta
CHOP	CCAAT-enhancer-binding protein homologous protein
CLF	Clofoctol
CReP	Constitutive Repressor of eIF2α Phosphorylation
CRE	CREB-responsive element
PKR	Double-stranded RNA-dependent protein kinase
ER	Endoplasmic reticulum
EBNA	Epstein-Barr nuclear antigen
EBV	Epstein–Barr virus
EBER	Epstein-Barr virus (EBV)-encoded small RNAs
eIF	eukaryotic Initiation Factor
GCN	General control non-depressible protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GFP	Green fluorescent protein
GADD	Growth arrest and DNA damage-inducible protein
HRI	Heme-regulated eIF2α kinase
HSV	Herpes simplex virus
HCMV	Human cytomegalovirus
HHV	Human herpesvirus
HIV	Human immunodeficiency virus
IRE	Inositol-requiring enzyme
ISR	Integrated stress response
ISRIB	Integrated stress response inhibitor
IE	Intermediate early
IRES	Internal ribosome entry site
JNK	c-jun N-terminal kinase
KSHV	Kaposi's sarcoma-associated herpesvirus
LMP	Latent membrane protein
LCL	Lymphoblastoid cell line
MHC	Major histocompatibility complex
MTOR	Mammalian target of rapamycin
MAPK	p38 mitogen-activated protein kinase

MEF	Myocyte-specific enhancer factor
NPC	Nasopharyngeal carcinoma
Poly(I:C)	Polyinosinic:polycytidylic acid
PCR	Polymerase chain reaction
PTLD	Post-transplant lymphoproliferative disorder
PERK	Protein kinase RNA-like endoplasmic reticulum kinase
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
NaB	Sodium butyrate
TPA	Phorbol ester
TRIB	Tribbles homolog
TM	Tunicamycin
UPR	Unfolded protein response
UTR	Untranslated region
uORF	upstream Open reading frame
XBP	X-box binding protein 1
ZRE	Zta response elements

INTRODUCTION

EBV

Epstein–Barr virus (EBV) is a virus of the gamma-herpes virus family and is also called human herpesvirus 4 (HHV-4). The double stranded DNA genome consists of 172kb and encodes more than 80 gene products [1]. The viral genome is surrounded by a nucleocapsid and viral envelope, which forms a viral particle. EBV infects mostly B cells and epithelial cells [2] and the initial Infection with EBV is thought to occur by contact with oral secretions [3]. Approximately 90% of the human population is infected with EBV. Infection with EBV usually occurs at early ages without symptoms and persists within the body in resting memory B cells as a latent infection [4]. Viral infection in adolescents and adults frequently results in infectious mononucleosis [5]. EBV is also associated with various types of cancers including nasopharyngeal carcinoma [6], Burkitt lymphoma [7] and Hodgkin lymphoma [8]. Patients with severe combined immunodeficiency, recipients of transplants, and patients with the acquired immunodeficiency syndrome (AIDS) have high incidence of EBV-associated lymphoproliferative disease, since their impaired T-cell immunity is incapable of controlling the proliferation of EBV-infected B cells [2, 9, 10].

Latent infection

EBV infection in B cells *in vitro* induces cell proliferation and can generate immortalized B cell lines called Epstein-Barr virus-transformed lymphoblastoid cell lines (LCL) [2]. Once the virus infects cells, EBV exists in two states - lytic and latent [11]. Unlike in lytic state where viral replication is followed by infectious virion production, the virus mostly exists in a latent state with restricted viral gene expression. There are 9 latent viral proteins in total. During the latency period, the viral genome circularizes and is maintained as an extrachromosomal episome and expression of viral protein EBNA1 is enough to replicate the episome by tethering it to the host chromosome, thus enabling episomal replication by cellular enzymes in dividing cells [12, 13]. EBNA2 is a main contributor for EBV driven cell growth by transcriptionally regulating viral and host protein expression. EBNA2 has been shown to bind CBF1/RBPJk and mimics the notch intracellular domain in the notch signaling pathway [14, 15]. LMP1 and LMP2A are the key latent membrane proteins that act as constitutively active receptors, which drive the signaling pathway in a ligand-independent manner [16, 17]. LMP1 is a functional homolog to CD40 [18] and LMP2A mimics B cell receptor signaling which is essential for the B cell survival [17]. Depending on cell type and differentiation stage of the B cell, EBV latency patterns can be categorized into three types of latency programs. The virus only expresses EBNA1 and some EBERs in latency 1, and with additional LMP1 and LMP2A expression, it becomes latency 2 which is typically shown in epithelial cells. All EBV latent genes are expressed during latency 3 and EBV- immortalized lymphoblastoid cell lines (LCL) are one example of B cell lines maintained with the type 3 pattern [19].

Lytic infection

The viral lytic phase is when virus replication occurs and the entire lytic gene repertoire is expressed producing infectious viral particles. This is rarely detected in immunocompetent humans as a result of being eliminated immediately by the cytotoxic immune response [20]. However, in the case of immunosuppressed patients, an abnormally increased level of infectious viral particles found in plasma can cause cell-to-cell transmission, resulting in a high incidence of EBV-induced lymphoproliferative disorders (LPD) [21]. Unlike the latent genes, lytic genes are expressed in a temporally regulated manner. The first gene that appears is the viral immediate early gene BZLF1, which encodes for Zta (or Z) protein that acts as a transcription factor regulating delayed early lytic gene expression to enable the viral DNA replication. Following this is viral late gene expression, which mostly encodes viral structural proteins in order to generate infectious virion particles.

EBV reactivation

The switch from latent infection to lytic infection is called EBV reactivation [22]. Although the precise mechanism of viral reactivation from the latent state *in vivo* is not completely known, lytic infection in cell culture systems has widely been investigated. After the first discovery of Z (Zta, or BZLF1) initiating viral lytic infection in latently infected cell lines [23, 24], there has been extensive investigations of the cellular regulation of the Z promoter and many chemical agents to induce lytic infection have been revealed and developed. For example, cross-linking the B-cell receptor with anti-immunoglobulin was used to induce lytic cycle in Akata cell lines [25] and other chemical agents such as phorbol esters, TPA [26], and calcium ionophores [27] were all shown to activate Z transcription by their effects on specific cellular pathways.

The Z promoter has two types of cis acting motifs – ZI and ZII, which are important for activating the promoter in response to various lytic inducing stimuli [28]. The ZI motif plays a dual role as a negative regulator and positive regulator of Z promoter activity. In the absence of lytic inducing stimuli, the ZI motif binds to a cellular protein MEF2D that represses Z transcription [29]. However, virus lytic inducing agents such as phorbol esters and anti-IgG prevent this interaction and convert it to a positive regulator for activating the promoter [30, 31].

The ZII motif is a CREB-responsive element (CRE) and is the other essential part for Z transcription [32]. Various cellular transcription factors such as c-jun/c-fos (AP1) [33, 34], CCAAT/enhancer-binding proteins (C/EBP) [35], and a spliced form of X-box-binding protein 1 (XBP1s) [36, 37] were found to act on the ZII motif to enhance the Z

transcription. The activation of transcription factors which bind to the ZII motif are typically mediated by the phosphorylation of cellular kinases such as c-jun N-terminal kinase (JNK) and p38 mitogen-activated protein kinase (MAPK) [34]. Once Z is activated by the cellular factors, the Z product is able to autoactivate itself by binding to another ZIII motif (ZRE site) and expression of Zta protein is enough to switch from latent to lytic cycle [38-40]. The initial expression of Z drives the expression of the other viral intermediate early (IE) gene, BRLF1 [41, 42] and both work together to activate most of the delayed lytic genes [43-45].

EBV associated cancers

EBV is well known as the causative virus for several cancers such as Burkitt lymphoma, post-transplant lymphoproliferative disorder (PTLD) and nasopharyngeal carcinoma (NPC).

Burkitt lymphoma is where the virus was first discovered in tumor cells [2]. EBV is thought to have a causal role in African Burkitt lymphoma as EBV is found in most cases and the majority of tumor cells harbor the viral genome [2]. Burkitt lymphoma is characterized by a chromosomal translocation of the c-myc oncogene near the immunoglobulin region, which drives upregulation of c-myc gene expression [46]. Overexpression of c-myc was shown to increase tumorigenicity in EBV-transformed lymphoblastoid cell lines.(LCL) [47].

Post-transplant lymphoproliferative disorder (PTLD) is another type of cancer highly associated with EBV [21]. This occurs after solid organ or bone marrow transplantation and approximately 50–80% of PTLD biopsies are positive for EBV within the tumor cells [48]. It is suspected that a weakened immune system allows EBV to proliferate the malignant cells.

There are also epithelial cancers associated with EBV, such as nasopharyngeal carcinoma (NPC). NPC is mostly found in southern China and is among the most common cancers in the area, where the incidence is about 50 per 100,000 persons every year [6]. It is thought that EBV infection in single cell initiates the development of tumor growth as clonal EBV DNA is found in preinvasive lesions of the nasopharynx [49]. EBV genome and proteins are also found in certain types of gastric carcinomas such as

undifferentiated lymphoepithelioma-like carcinomas and is thought to have a pathogenic role in those tumors [50].

EBV targeted therapy

The presence of the EBV genome in tumors offers a potential advantage as it serves as a tumor-specific target. The strategies for EBV-targeted therapies have been implemented for various types of EBV-associated cancers. One of these strategies is using an anti-viral nucleoside analog prodrug, ganciclovir, which is selectively phosphorylated by viral kinases to become the active cytotoxic form. In this case, viral lytic induction to enhance viral kinase expression would target EBV-harboring cells exclusively. Epigenetic modulators and some anti-cancer chemotherapy drugs were shown to induce lytic activation and have a killing effect with ganciclovir in various types of EBV cancer cell lines [51-53] as well as in a clinical trial [54]. However, the evidence suggesting that tumor killing in these cell lines or in this clinical trial is truly EBV-specific remains largely conjectural. Further research revealed several other cellular mechanisms that result in Zta induction including ER stress and the ATM/p53 pathway during genotoxic stress response [55, 56] and clinical drugs that elicit the pathways were proven to have an effect on EBV lytic gene expression and a cytotoxic effect together with antiviral nucleoside analogs *in vitro* and *in vivo* [57, 58].

EBV-specific cytotoxic T lymphocytes (EBV CTL) therapy was developed for immunocompromised patients where a suppressed T cell response to EBV infected cells is thought to drive tumor development [59]. These types of tumors, such as post-transplant-lymphomas, typically show a type III latency pattern which expresses the full spectrum of latent viral genes and are most readily targeted. Viral lytic genes are also readily targeted by normal host T cell responses.

CHAPTER 1: CLOFOCTOL INDUCES EBV LYTIC ACTIVATION IN MULTIPLE CELL LINES

SUMMARY

Epstein-Barr virus (EBV) is a human gammaherpesvirus which is responsible for causing various types of cancer. The unfolded protein response (UPR) is one of the major pathways which leads EBV to enter the lytic replication cycle. Thus several UPR inducers such as bortezomib are widely used for activating EBV in various cancer cell lines in laboratory [60]. Recent investigations have demonstrated that an antibacterial drug, clofoctol, which has anti-proliferative activities in prostate cancer cell lines, activates the UPR [64]. As it was previously reported that bortezomib induces EBV lytic gene expression through the UPR pathway, we investigated whether clofoctol had an effect on the activation of EBV in latently infected cancer cell lines. Here, we show that clofoctol leads to EBV activation in lymphoma cell lines, epithelial cancer cell lines and lymphoblastoid cell lines. Thus clofoctol activates a broader range of EBV cell lines than does bortezomib. Increased EBV genome copy number per cell resulting from clofoctol treatment was demonstrated by qPCR. Clofoctol also increased viral immediate early lytic gene Zta, early lytic gene Bmrf1, and late lytic gene gp350 RNA expression as demonstrated by qRT-PCR. In the BX1-Akata Burkitt lymphoma cell line, increased viral DNA copy number (as measured by marker GFP expression) and increased Zta protein expression was shown by immunofluorescence and immunoblot. In contrast, clofoctol treatment did not increase gp350 protein expression, a late viral protein. Furthermore, in a Raji infection assay that utilizes GFP-marked EBV, it was clear that clofoctol treatment blocked infectious virion production.

BACKGROUND

Epstein–Barr virus (EBV), a human gammaherpesvirus, is a cancer-associated virus and it is mostly in the latent state in cancer cells. Physiological stimuli that induce the lytic cycle *in vivo* are yet not clear but some reagents have been known to trigger viral reactivation *in vitro*. There has been growing interest in pharmacologic activation of lytic viral gene expression in EBV (+) tumor tissue and several recent therapeutic strategies require activation of viral gene expression to achieve tumor regression [61]. One of reagents studied for the lytic induction strategy is bortezomib, a clinically approved proteasome inhibitor used for multiple myeloma treatment. A previous report showed that bortezomib induces EBV lytic replication by inducing the unfolded protein response (UPR) *in vitro* [60] and the effect was also studied in an *in vivo* tumor model system [57].

In a recent study, the antibiotic drug clofoctol has been shown to inhibit the growth of prostate cancer cells at clinically achievable concentrations [62, 63], which was mediated by activation of UPR pathways [64]. Clofoctol ([2-(2,4-dichlorobenzyl)-4-(tetramethyl-1,1,3,3-butyl)phenol]) is a synthetic antibacterial drug which is effective on various gram-positive and gram-negative bacteria. This drug has been widely used in France and Italy for treating mild upper respiratory tract infections and is well-tolerated in patients [65, 66]. While the exact mechanism of action is not completely understood, it has been suggested to act on the cytoplasmic membrane of bacteria and inhibit cell wall synthesis [67]. Based on previous evidence showing that EBV Zta expression can be activated by pharmacologic activation of UPR pathways, we sought to examine the effect of clofoctol on EBV lytic activation.

There are several types of cancer cell lines that harbor EBV. The Akata cell line was derived from a Burkitt lymphoma patient and the BX1-Akata cell line is an engineered derivative cell line that carries recombinant EBV expressing GFP [68, 69]. Treatment with lytic inducing agents such as anti-immunoglobulin drive the viral lytic cycle, which can be visualized by enhanced GFP expression. C666 is a nasopharyngeal carcinoma cell line [70] and SNU719 is a gastric carcinoma cell line, both of which are derived from tumors naturally infected with EBV [71]. A lymphoblastoid cell line (LCL) is a B cell line that was immortalized by EBV *in vitro.* All of the above cell lines are predominantly latently infected and responses to lytic inducers vary among the cell types [72].

Lytic induction can be monitored by several types of laboratory techniques. Quantitative polymerase chain reaction (qPCR) analysis is used to measure the copy number of EBV genomes. A convenient EBV standard is DNA from the Namalwa Burkitt lymphoma cell line that has 2 copies of integrated EBV genome per cell [73]. EBV lytic RNA expression can also be measured by quantitative reverse transcribed polymerase chain reaction (qRT-PCR). EBV lytic proteins can be measured using immunoblot or immunofluorescence. The first lytic gene expressed is the EBV immediate early lytic gene BZLF1 which encodes for Zta. This induces the other immediate early lytic gene BRLF1 expression and both drive the serial expression of early lytic genes. The majority of viral early lytic genes encode enzymes and proteins that work for efficient viral DNA replication, such as the viral DNA polymerase processivity factor, Bmrf1 gene. Viral genome replication is followed by viral late gene expression, which encodes viral structural proteins, such as gp350 encoding for the viral membrane

glycoprotein. Finally the replicated viral DNA genome is incorporated into the viral capsid structure and the nucleocapsids are enveloped with the tegument proteins and glycoprotein-rich membrane, which results in viral particle formation [74].

By using various laboratory techniques, we investigated the effects of clofoctol on EBV lytic activation in multiple cancer cell lines.

MATERIALS AND METHODS

Cell Culture. Akata and Raji are EBV (+) Burkitt lymphoma cell lines. BX1-Akata, an engineered derivative Akata line which carries a recombinant EBV that constitutively expresses a green fluorescent protein (GFP), was a gift from L. Hutt-Fletcher (Louisiana State University). SNU-719, a naturally derived EBV (+) gastric cancer cell line, was a gift from J. M. Lee (Yonsei University). C666-1 is an EBV (+) nasopharyngeal carcinoma cell line and LCL is an EBV-immortalized lymphoblastoid cell line. All cell lines were cultured in RPMI 1640, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin and 10% v/v fetal bovine serum (FBS). 500 μg/mL G418 (geneticin; Life Science Technologies) was added to the culture medium for the BX1-Akata cell line.

Reagents. Clofoctol was a gift from J. Liu (Johns Hopkins University School of Medicine) and purchased from Sigma-Aldrich. Bortezomib (20 nM) was from Millennium Pharmaceuticals, tunicamycin (2 μg/mL) was from Enzo Life Sciences, anti-IgG (10 μg/mL) was from Sigma-Aldrich.

qPCR. DNA was extracted from cells using a QIAamp DNA mini kit (QIAGEN). Real-time PCR was performed with EBV BamH-W primers and the BamH-W fluorescent probe. BamH-W Forward (5'-CCCAACACTCCACCACACC-3'), BamH-W Reverse (5'-TCTTAGGAGCTGTCCGAGGG-3') and the BamH-W fluorescent probe (5'-(FAM)CACACACTACACACACCCACCCGTCTC(BH1)-3') were used. EBV copy numbers were normalized compared to serially diluted Namalwa cell DNA. Each reaction was 20 μL in total, including 2 μL DNA at 50 ng/mL, 2 EBV primers at 500 nM, the

probe at 200 nM and SsoAdvance Supermix (Bio-Rad). DNA was amplified at 95°C for 2 minutes for 1 cycle and 95°C for 5 seconds and 60°C for 10 seconds, for total 40 cycles in a CFX96 real time thermocycler (Bio-Rad).

qRT-PCR. RNA was extracted using an RNeasy Mini Kit (QIAGEN) and reverse-transcribed into cDNA by using iScript reverse synthase kit (Bio-Rad). Reverse transcription was performed using a CFX96 real time thermocycler (Bio-Rad). SsoFast Evagreen Supermix (Bio-Rad) with 500 nM primers and cDNA corresponding to 25 ng of the RNA was used for each reaction. cDNA was amplified at 95°C for 30 seconds for 1 cycle and 95°C for 5 seconds and 60°C for 10 seconds, for a total of 40 cycles in a CFX96 real time thermocycler. EBV Zta primers used were Forward (5'-ACATCTGCTTCAACAGGAGG-3') and EBV Zta Reverse (5'-AGCAGACATTGGTGTTCCAC-3'). EBV Bmrf1 primers used were Forward (5'-CTAGCCGTCCTGTCCAAGTGC-3') and EBV Bmrf1 Reverse (5'-AGCCAAACGCTCCTTGCCCA-3'). EBV gp350 primers used were Forward (5'-GTCAGTACACCATCCAGAGCC-3') and EBV gp350 Reverse (5'-TTGGTAGACAGCCTTCGTATG-3'). GAPDH primers were used as a control for normalization. GAPDH primers used were Forward (5'-TCTTTTGCGTCGCCAGCCGA-3') and GAPDH Reverse (5'-AGTTAAAAGCAGCCCTGGTGACCA-3').

Immunoblots. For protein extractions, 1.5 x 10^7 cells were washed in PBS and the pellets were resuspended in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 100 μM EDTA and 1X protease/phosphatase inhibitor cocktail (Cell Signaling Technologies). After 15 minutes incubation in ice, 0.6% NP-40 was added and vortexed

for cell lysis. The cytosolic proteins were separated by centrifugation at 10,000 rpm for 30 seconds and collected by removing the supernatant. The pellet containing the nuclear proteins was resuspended in a buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA and 1X protease/phosphatase inhibitor cocktail (Cell Signaling Technologies). After 15 minutes of rotation at 4°C, the nuclear proteins were isolated by centrifugation at 13,000 rpm at 4°C for 5 minutes and collected by removing the supernatant. SDS-PAGE and Western blotting were performed with equal amounts of proteins per sample and ECL chemiluminescent detection reagents (GE Healthcare) were used in conjunction with autoradiography film (Denville Scientific) detection. Antibody against EBV Zta was from Santa Cruz Biotechnology and anti- β actin was from Sigma-Aldrich.

Immunofluorescence. 1.5×10^5 cells were spun onto microscope slides by using a Cytospin centrifuge, fixed and permeabilized with ice-cold methanol for 15 minutes, blocked in PBS with 5% Bovine serum albumin (BSA) for 30 minutes, incubated with anti-EBV Zta or gp350 mouse antibody (Santa Cruz Biotechnology) at 1:50 for 1 hour, washed three times for ten minutes each with 5% BSA, 0.1% Tween-20 in PBS and Cy3 goat anti-mouse antibody (Jackson Immunoresearch) was applied to the cells for 1 hour at room temperature. After 3 final washes, the cells were stained with Vectashield mounting media with DAPI (Vector Laboratories). A ZOE Fluorescent cell imager (Bio-Rad) was used for cell fluorescence detection.

Raji infection assay. BX1-Akata cells were treated with anti-immunoglobulin G (MilliporeSigma, 10 μg/ml), TPA (20 ng/ml)/NaB (3 mM) or clofoctol (20 uM) and incubated for 4 days. After spinning the cells, the supernatant was passed through a

Millex-HV Syringe Filter Unit (0.45 μm, MilliporeSigma) and concentrated with a centrifugal filter (Amicon Ultra-15 Centrifugal Filter Unit, MilliporeSigma). Raji cells were infected with the concentrated cell free virus and TPA/NaB were added 24 hours after the infection. The GFP positive cells were counted 24 hours after TPA/NaB treatment using fluorescence microscopy.

RESULTS

We previously reported that bortezomib induces EBV lytic gene expression through the UPR pathway. In this vein, we investigated whether clofoctol can induce the activation of EBV in latently-infected lymphoma cell lines. As shown in Figure 1-1, treatment of clofoctol increased the expression of EBV immediate early gene Zta RNA in BX1-Akata, Akata EBV (+) and Raji cells and an increased Zta protein level was also found by immunoblot. We also confirmed that further rounds of viral lytic gene expression were upregulated by clofoctol, as assessed by qRT-PCR for the early lytic gene Bmrf1 and the late EBV envelope glycoprotein gene gp350 (Fig. 1-2). The increased level was similar to that seen with bortezomib. We also observed a dose-dependent increase of EBV viral DNA level in BX1-Akata cells with clofoctol treatment (Fig. 1-3). Green fluorescent protein (GFP) expression in the BX1-Akata cell line serves as an indicator of EBV lytic replication, and anti-IgG treatment results in an increase of GFP expression. The number of GFP (+) cells in the BX1-Akata cell line was increased with clofoctol treatment as shown in Figure 1-3b. We further confirmed the increased Zta protein expression by performing an immunofluorescence assay. However, clofoctol treatment did not increase late lytic gene gp350 protein expression (Fig. 1-4).

EBV is also associated with epithelial cell cancers such as gastric carcinoma and nasopharyngeal carcinoma (NPC). Thus we tested the effects of clofoctol on EBV in C666, a native EBV (+) NPC cell line and in SNU-719, a native EBV (+) gastric carcinoma cell line. Like EBV-harboring lymphoma cells, the EBV DNA level as well as Zta RNA level were all increased by clofoctol treatment in C666 and SNU-719 cells (Fig.

1-5). Based on these results, we conclude that clofoctol induces EBV lytic gene expression in naturally infected EBV (+) epithelial cancer cell lines.

Although bortezomib is a potent EBV lytic inducer, it has limited effects on lytic activation in EBV-immortalized lymphoblastoid cells (LCLs). We tested clofoctol in LCLs to see whether latently infected EBV in LCLs can be reactivated by clofoctol treatment. As shown in Figure 1-6, we found a strong effect in activating virus in LCLs, which was measured by increased expression of EBV Zta, Bmrf1 and gp350 RNA. Clofoctol treatment increased the number of Zta protein expressing cells in a similar level to the RNA increase. These results indicate that clofoctol induces EBV lytic activation in LCLs and is likely to have a distinct mechanism for this induction.

We further investigated whether EBV lytic gene expression and lytic replication induced by clofoctol lead to enhanced infectious virion production. The virus producer BX1-Akata cell line was treated with clofoctol as shown in Figure 1-7a, and the cell-free BX1 virus supernatants were used to perform a Raji cell infection assay. We found that no GFP (+) Raji cells were detected in samples with clofoctol-induced BX1 virus, which indicates that clofoctol treatment blocks infectious virion production.

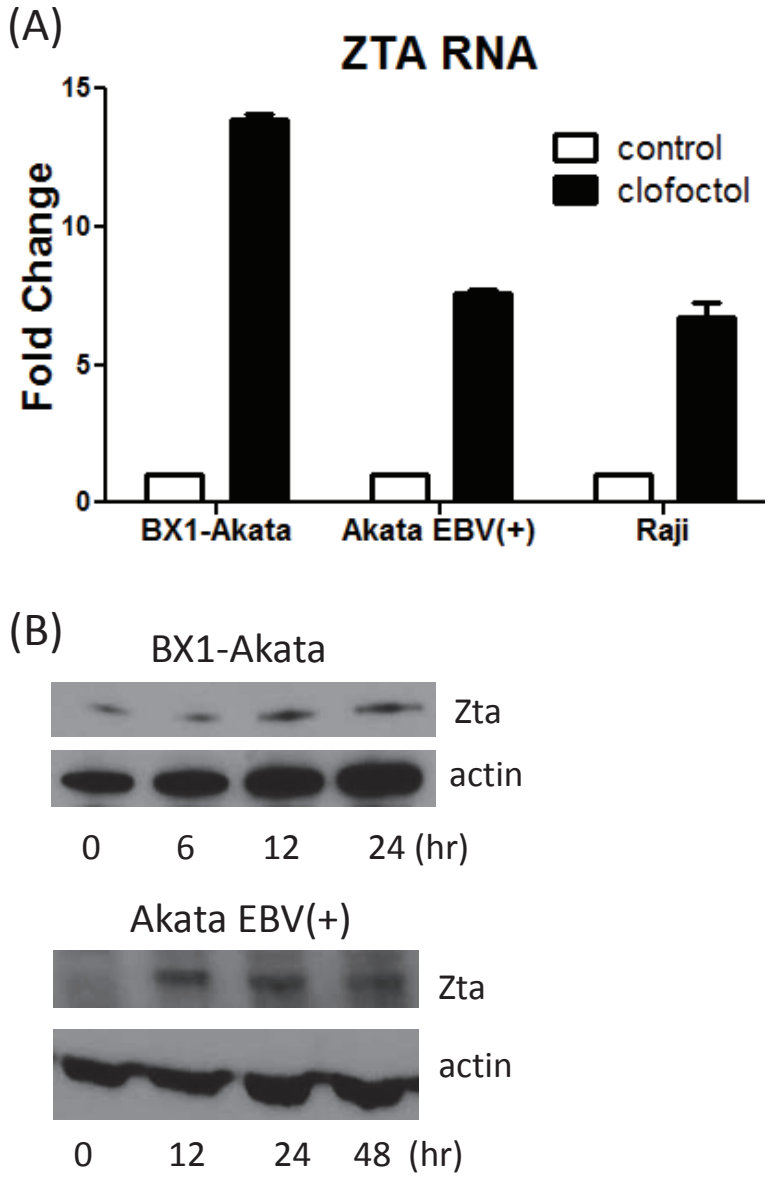


Figure 1-1. Clofoctol activates EBV Zta expression in lymphoma cell lines

A. BX1-Akata, Akata EBV(+) and Raji cells were treated with clofoctol 20 uM for 24 hours and isolated RNA were used to perform qRT-PCR for detecting Zta RNA levels.

B. Expressions of Zta proteins were measured by western blot at indicated time points 24 hours after the treatment in BX1-Akata and Akata EBV(+) cells.

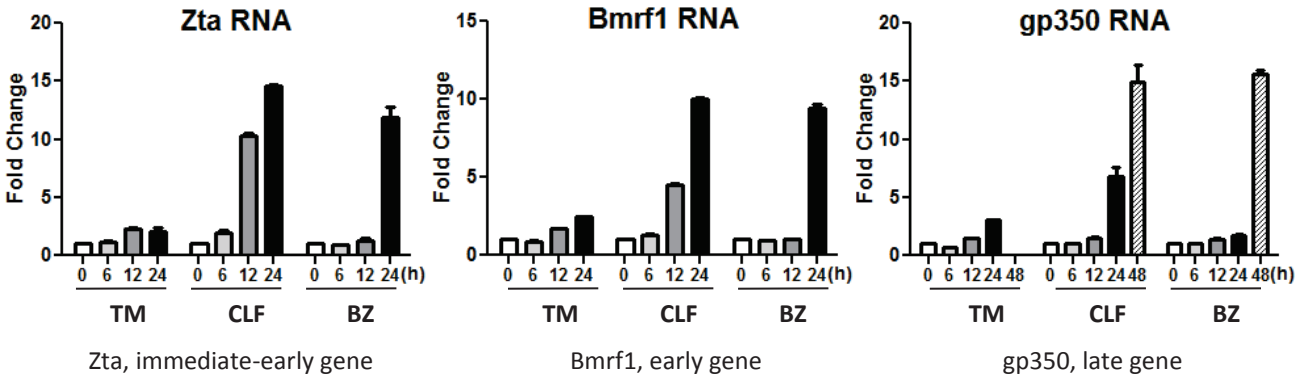


Figure 1-2. Clofoctol activates EBV lytic gene expression in BX1-Akata cells

BX1-Akata cells were treated with tunicamycin (TM, 2 μg/mL), clofoctol (CLF, 20 uM) or bortezomib (BZ, 20 nM) and RNAs were isolated at the time point to perform qRT-PCR to detect Zta, Bmrf1 and gp350 transcript levels.

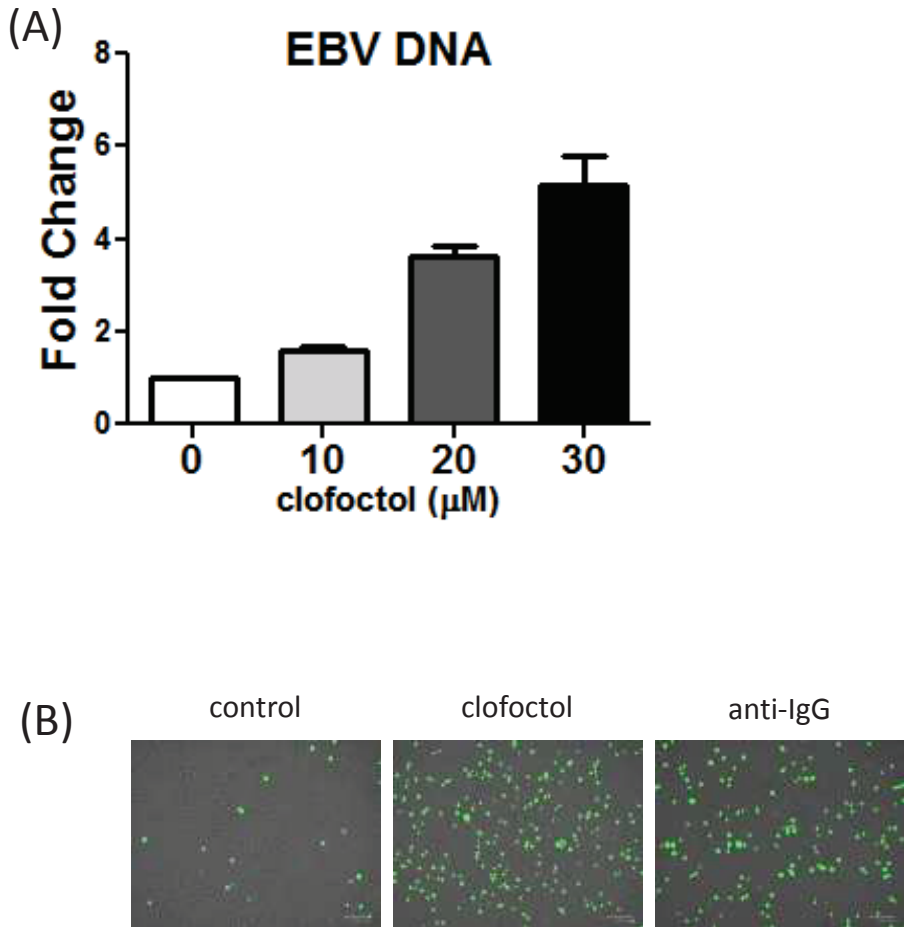


Figure 1-3. Clofoctol induces EBV lytic replication in BX1-Akata cells

A. BX1-Akata cells were treated with indicated doses of clofoctol. 24 hours later, DNA was isolated and q-PCR was performed with EBV BamW primers

B. BX1-Akata cells were treated with 20 uM clofoctol or anti-IgG for control. Fluorescence microscopy was used to determine the number of cells expressing GFP 24 hours after the treatment.

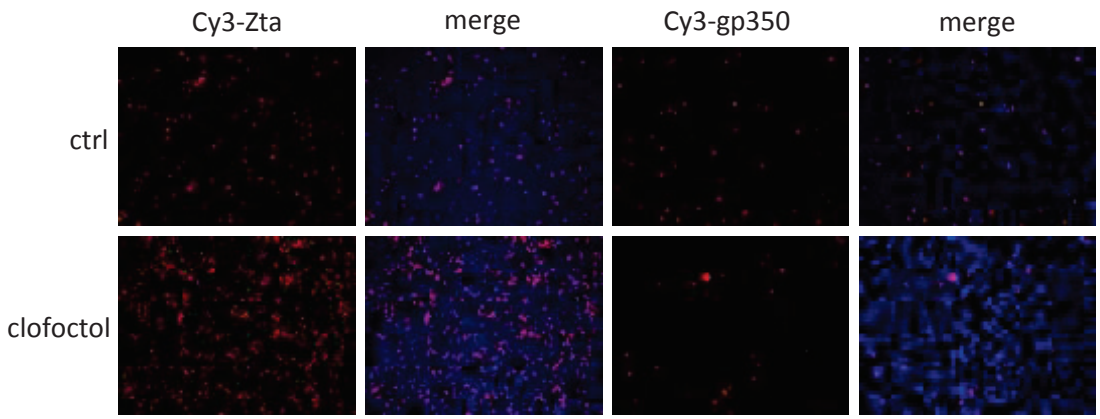


Figure 1-4. Clofoctol induces Zta, but not gp350 protein expression

Expression of Zta and gp350 proteins were measured by immunofluorescence 24 hours after the clofoctol (20 uM) treatment in BX1-Akata cells.

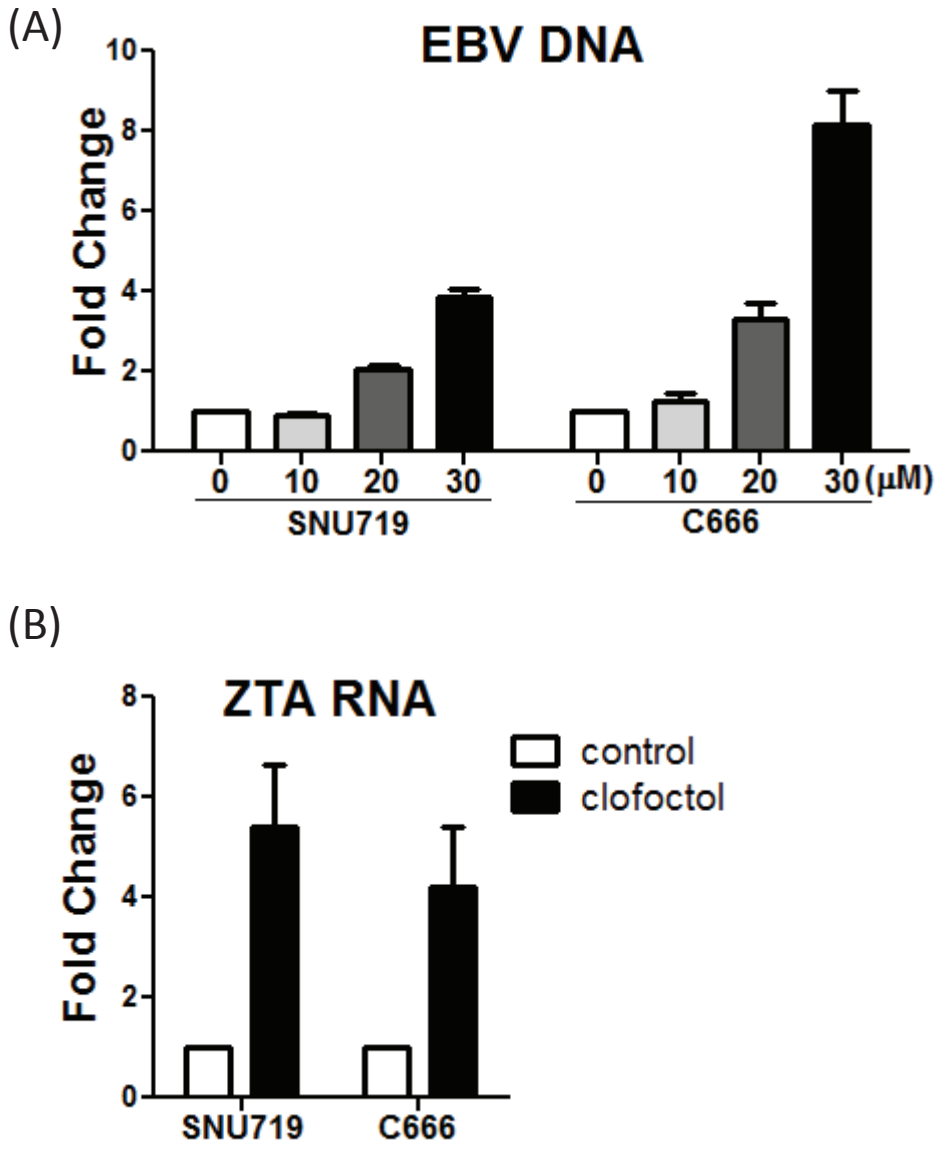


Figure 1-5. Clofoctol activates lytic viral infection in carcinoma cells

SNU-719 and C666 cells were treated with indicated doses of clofoctol for 24 hours. DNA was isolated for EBV DNA quantification by q-PCR (A) and RNA was also isolated for Zta mRNA quantification by qRT-PCR (B).

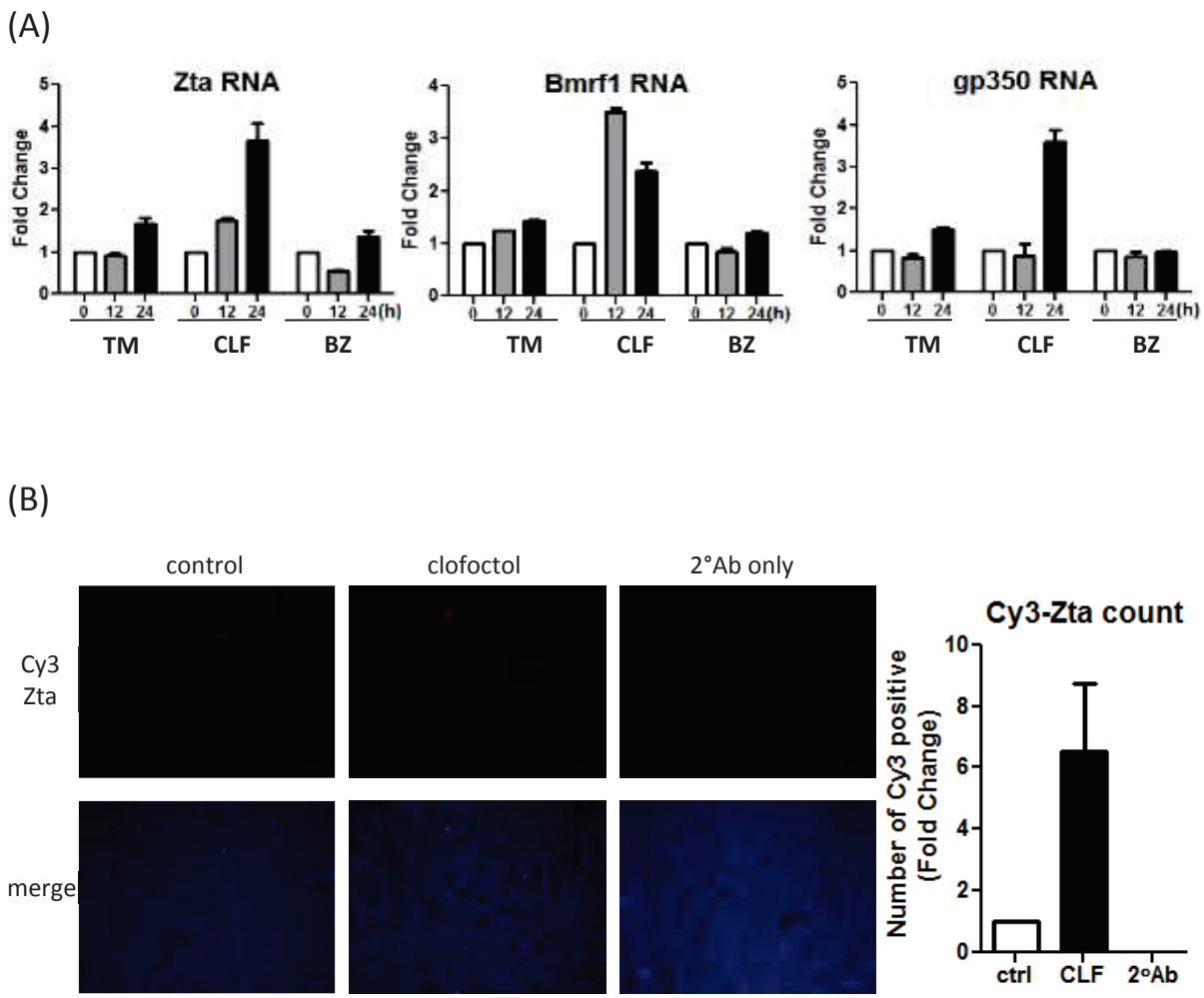
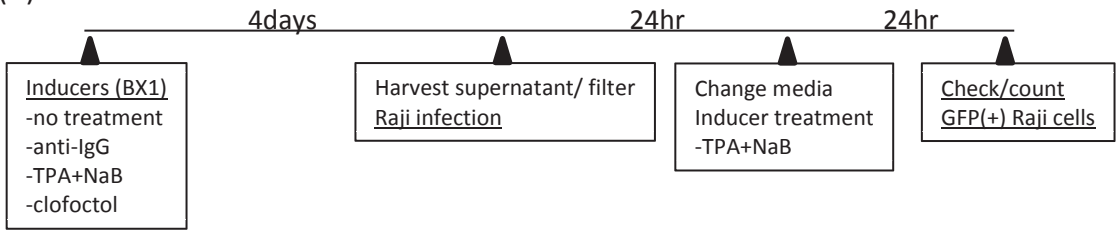


Figure 1-6. Clofoctol induces EBV lytic activation in EBV-immortalized lymphoblastoid cells

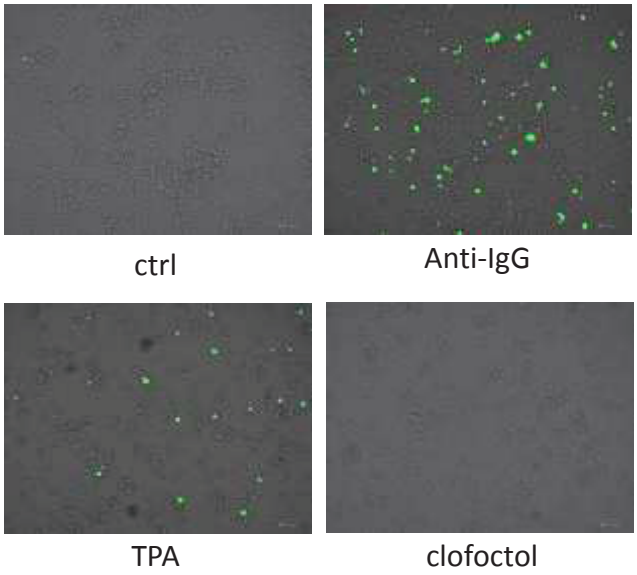
A. LCLs were treated with tunicamycin (2 µg/mL), clofoctol (20 uM) or bortezomib (20 nM) and RNA was isolated at the time point to perform qRT-PCR to detect Zta, Bmrf1 and gp350 transcript levels.

B. LCLs were treated clofoctol 20 uM for 24 hours. Expression of Zta protein was measured by immunofluorescence and the number of cy3-Zta expressing cells were counted in triplicate and compared to the untreated sample.

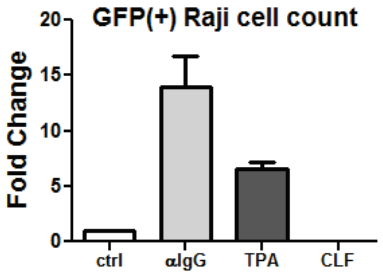
(A)


4days
24hr
24hr
Inducers (BX1)
-no treatment
-anti-IgG
-TPA+NaB
-clofoctol
Harvest supernatant/ filter
Raji infection
Change media
Inducer treatment
-TPA+NaB
Check/count
GFP(+) Raji cells

(B)


ctrl
Anti-IgG
TPA
clofoctol

(C)


GFP(+) Raji cell count
Fold Change
20
15
10
5
0
ctrl
αIgG
TPA
CLF

(D)

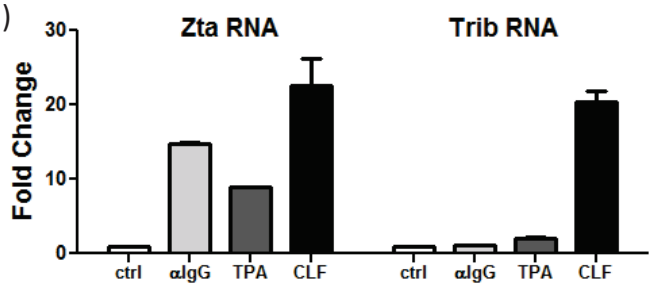

Zta RNA
Trib RNA
Fold Change
30
20
10
0
ctrl
αIgG
TPA
CLF
ctrl
αIgG
TPA
CLF

Figure 1-7. Clofoctol inhibits virion production

A. Raji cell infection assay was performed to determine infectious viral titers according to the indicated schedule.

B and C. Raji cells were infected with the cell-free BX1 virus supernatants of each drug treatment and GFP (+) Raji cells were imaged (B) and counted (C).

D. BX1-Akata cells were treated with anti-immunoglobulin G (10 μg/ml), TPA (20 ng/ml) or clofoctol (20 uM) for 24 hours and isolated RNAs were used for quantification of Zta and the ATF4 target gene Trib transcript levels.

DISCUSSION

EBV lytic induction by bortezomib has been well characterized and here we confirmed the earlier report of bortezomib inducing EBV lytic gene expression in Burkitt cell lines. Many reports also showed that bortezomib produced its clinical activity in various types of gammaherpesvirus associated cancers [57, 75-77]. Our results suggest that clofoctol acts very similarly to bortezomib in that EBV lytic gene expression was similarly increased in BX1-Akata cells, although the effect was more rapid than that of bortezomib. It was previously reported that bortezomib has cytotoxic effects against LCL without any effect on Zta induction [78]. Here we confirmed that bortezomib treatment of LCL does not drive any EBV lytic gene expression. However, clofoctol works as a potent lytic activator in LCL, as evidenced by increased RNA expression of Zta, Bmrf1, and gp350, and enhanced Zta protein expression. Clofoctol was also active in naturally infected EBV-epithelial cancer cell lines, SNU719 and C666, as viral DNA copy number increased in a dose-dependent manner.

Interestingly, although all EBV lytic gene RNA expression as well as the IE Zta protein expression was increased by clofoctol treatment, late viral gp350 protein expression did not increase, nor did infectious virion production. As viral glycoproteins are essential for infectious virus production as well as the viral entry into cells [79], the repressed late viral protein expression presumably inhibited virion production. Previously, UPR-mediated inhibition of infectious virion production was studied with HCMV [80]. The report showed that the activation of the UPR by thapsigargin and clotrimazole significantly inhibits infectious virion production, which was attributed to the retarded accumulation of late gene products and the drug-induced disruption of virion maturation

by depleted ER calcium. A similar finding was reported with HSV, which demonstrated that an HIV protease inhibitor, Nelfinavir, inhibited HSV-1 viral replication by affecting viral glycoprotein maturation [81]. Recently, investigators discovered that several other drugs such as spironolactone, a mineralocorticoid receptor antagonist, and chloroquine, an antimalarial drug, inhibit EBV virion production without affecting lytic viral DNA replication [82, 83].

Similar to clofoctol in this report, other EBV lytic inducers that work through the UPR, such as bortezomib and nelfinavir, do not lead to infectious virion production (unpublished data), which indicates a common feature of the UPR-mediated lytic mechanism. As clofoctol has been used as a drug in the clinic for a decade with tolerable side effects, clinical trials could be performed to confirm the anticipated clinical benefit of the treatment of gammaherpesvirus-associated cancers and further studies to elucidate the specific mechanism as well as to find its direct target will provide better insight into clofoctol action.

CHAPTER 2: THE EFFECTS OF CLOFOCTOL ON THE UNFOLDED PROTEIN RESEPONSE

SUMMARY

As clofoctol was shown to activate the unfolded protein response (UPR) in a prostate cancer cell line (PC3) [64], we investigated the effects of clofoctol on the UPR in BX1-Akata Burkitt lymphoma cells and LCL. There are three branches in the UPR which are activated by each of the ER transmembrane proteins - PERK, IRE1 and ATF6 [84].

One branch of the UPR involves PERK, which phosphorylates eIF2α leading to a rapid attenuation of protein synthesis but facilitating translation of the transcription factor ATF4 and certain other RNAs. ATF4 upregulates the expression of proteins that diminish ER stress, including CHOP. We found that clofoctol increased the level of phosphorylated eIF2α, up-regulated ATF4 and CHOP.

A second branch of the UPR involves processing of the transcription factor XBP1 so as to regulate expression of ER stress response genes. Activation of the UPR leads to splicing of XBP1 mRNA. Thus an increase in spliced XBP1 RNA (XBP1s) is indicative of ER stress. Using reverse transcriptase-PCR, we observed that clofoctol led to increased XBP1s.

The third branch of UPR involves the activation of ATF6 by proteases leading to translocation of the N-terminal fragment into the nucleus with activation of target genes such as Bip. With clofoctol treatment, we confirmed the ATF6 activation evidenced by Bip expression.

Here, we show that all branches of UPR were activated by clofoctol in BX1-Akata cells and the effect was similar to that seen with other UPR inducers, such as tunicamycin and bortezomib. Clofoctol also induces all three branches of UPR in LCL, where bortezomib has a limited effect.

BACKGROUND

The switch from latent infection to lytic infection depends on the level of Zta (BZLF1) expression. Therefore, the molecular basis of transcription on Z promoter has been widely investigated by using various types of chemical or biological inducers. In some B cell derived EBV (+) cell lines, mimicking the B cell receptor signaling by cross-linking with anti-immunoglobulin treatment is a potent activator of EBV and this finding prompted wide investigations into the regulation of the Z promotor by cellular signaling pathways [85].

Another cellular stimulus that induces viral reactivation is endoplasmic reticulum (ER) stress [56]. ER stress can be triggered by accumulation of misfolded proteins within the ER, which leads to the unfolded protein response (UPR) [60]. The UPR is a cellular compensatory mechanism in the ER, which plays an important role in restoring protein load and folding capacity [84]. It starts from three ER transmembrane sensors: Protein kinase RNA (PKR) - like ER kinase (PERK), inositol-requiring enzyme 1 (IRE1), and activating transcription factor 6 (ATF6) [86] and each activates three downstream UPR pathways to inhibit global protein translation and activate their target genes, which help with proper protein folding [87]. The first branch is mediated by phosphorylation of a subunit of eukaryotic initiation factor 2 α (eIF2α) by oligomerized double-stranded RNA-activated PK-like ER kinase (PERK), which preferentially translates activating transcription factor 4 (ATF4) to drive the expression of its target genes such as C/EBP homologues protein (CHOP). The next branch of UPR starts from oligomerized inositol requiring enzyme 1 (IRE1) in the ER lumen, which catalyzes splicing of X-box binding protein 1 (XBP1) mRNA, generating a spliced isoform of XBP-1 mRNA (XBP1s).

Lastly, the activating transcription factor 6 (ATF6) pathway is activated by cleavage of the N-terminal fragment of ATF6, which translocates into the nucleus and activates its target genes, such as Binding immunoglobulin protein (Bip) [88].

As clofoctol was shown to upregulate the UPR in a prostate cancer cell line (PC3) [64], we investigated the effect of clofoctol on the UPR in BX1-Akata Burkitt lymphoma cells as well as in LCL and compared with other well-studied UPR inducers such as tunicamycin and bortezomib. Tunicamycin, which inhibits the N-linked glycosylation required for proper protein folding, is widely used as an UPR inducer [89]. Bortezomib inhibits the 26S proteasome and the effect on the UPR was well studied in multiple myeloma cells and lymphoma cells [60, 90]. Activated UPR by bortezomib was shown to increase CCAAT/Enhancer Binding Protein (CEBP)/beta transcription, which facilitates its efficient binding to Zta promoter region [60].

MATERIALS AND METHODS

Cell Culture. BX1-Akata is an engineered derivative Akata line which carries a recombinant EBV that constitutively expresses a green fluorescent protein (GFP) and LCL is an EBV-immortalized lymphoblastoid cell line. All cell lines were cultured in RPMI 1640, 2 mM L-glutamine, 100 IU/mL penicillin, 100 µg/mL streptomycin and 10% v/v fetal bovine serum (FBS). 500 µg/mL G418 (geneticin; Life Science Technologies) was added to the culture medium for the BX1-Akata cell line.

Reagents. Clofoctol was a gift from J. Liu (Johns Hopkins University School of Medicine) and purchased from Sigma-Aldrich. Bortezomib (20 nM) was from Millennium Pharmaceuticals.

qRT-PCR. RNA was extracted using an RNeasy Mini Kit (QIAGEN) and reverse-transcribed into cDNA by using iScript reverse synthase kit (Bio-Rad). Reverse transcription was performed using a CFX96 real time thermocycler (Bio-Rad). SsoFast Evagreen Supermix (Bio-Rad) with 500 nM primers and cDNA corresponding to 25 ng of the RNA was used for each reaction. cDNA was amplified at 95°C for 30 seconds for 1 cycle and 95°C for 5 seconds and 60°C for 10 seconds, for total 40 cycles in a CFX96 real time thermocycler. GAPDH primers were used as a control for normalization. GAPDH primers used were Forward (5'-TCTTTTGCGTCGCCAGCCGA-3') and GAPDH Reverse (5'-AGTTAAAAGCAGCCCTGGTGACCA-3'). XBP1s primers used were Forward (5'- TGCTGAGTCCGCAGCAGGTG-3') and XBP1s Reverse (5'-GCTGGCAGGCTCTGGGGAAG-3'). CHOP10 primers used were Forward (5'-AAGATGAGCGGGTGGCAGCG-3') and CHOP10 Reverse (5'-ACCTGCTTTCAGGTGTGGTGATG-3'). Bip/GRP78 primers used were Forward (5'-

GTTCTTGCCGTTCAAGGTGG-3') and Bip/GRP78 Reverse (5'-TGGTACAGTAACAACTGCATG-3'). Trib(3) Forward (5'-CGTGATCTCAAGCTGTGTCG-3') and Trib(3) Reverse (5'-AGCTTCTTCCTCTCACGGTC-3').

Immunoblots. For protein extractions, 1.5 x 10^7 cells were washed in PBS and the pellets were resuspended in a buffer containing 10 mM HEPES (pH 7.9), 10 mM KCl, 100 μM EDTA and 1X protease/phosphatase inhibitor cocktail (Cell Signaling Technologies). After 15 minutes incubation in ice, 0.6% NP-40 was added and vortexed for cell lysis. The cytosolic proteins were separated by centrifugation at 10,000 rpm for 30 seconds and collected by retaining the supernatant and the pellet with the nuclear proteins was resuspended in a buffer containing 20 mM HEPES (pH 7.9), 400 mM NaCl, 1 mM EDTA and 1X protease/phosphatase inhibitor cocktail. After 15 minutes of rotation at 4°C, the nuclear proteins were isolated in the supernatant by centrifugation at 13,000 rpm at 4°C for 5 minutes. SDS-PAGE and Western blotting were performed with equal amounts of proteins per sample and ECL chemiluminescent detection reagents (GE Healthcare) were used for in conjunction with autoradiography film (Denville Scientific) detection. Antibodies against p-eIF2α and anti-eIF2α were from Abcam, anti-ATF4 and anti-C/EBPβ were from Santa Cruz Biotechnology, anti- β actin from Sigma-Aldrich, anti-ATF6 from Abcam and anti-XBP1s was from Biolegend.

RESULTS

As a previous report showed that clofoctol activates the unfolded protein response (UPR) in prostate cancer cell lines, we were interested in knowing whether clofoctol would have the same effects on EBV (+) lymphoma cells. As shown in Figure 2-1, the UPR consists of three branches. First, the PERK branch starts from activated PERK which phosphorylates eIF2α, resulting in increased ATF4 translation. ATF4 upregulates transcription of a variety of target genes including CHOP. We performed immunoblots to measure the level of phosphorylated eIF2α and ATF4, and qRT-PCR to measure CHOP RNA expression after clofoctol treatment of BX1-Akata cells. We found that clofoctol increased p-eIF2α and ATF4 protein as well as CHOP RNA (Fig. 2-2). The second branch of the UPR starts from the cleavage of ATF6. The N-terminal fragment of ATF6 works as a transcription factor for turning on the target's gene expression, such as Bip. Activation of the ATF6 branch by clofoctol was confirmed by immunoblot and qRT-PCR. In the last branch of the UPR, activated IRE1 mediates the splicing of XBP1. We found that clofoctol increased the level of spliced isoform of XBP1 as seen in both RNA and protein level (Fig. 2-2). These data suggest all three branches of UPR were activated by clofoctol in BX1-Akata cells.

To further investigate the effects of clofoctol on the UPR in LCL, we performed qRT-PCR to measure the RNA levels of two ATF4 target genes (CHOP and Trib), an ATF6 target gene (Bip) and XBP1s. We found that all three branches were activated by clofoctol in LCLs, just as in BX1-Akata cells. Conversely, bortezomib does not activate any of the UPR branches in LCLs (Fig. 2-3). This result suggests that the inability of bortezomib to induce viral activation in LCL may be due to the inactivation of UPR.

C/EBPβ is one of major transcription factors that can directly bind and contribute to EBV Zta promoter expression, and we previously reported that Zta promoter induction by bortezomib is mediated by increased C/EBPβ expressions [60]. We therefore examined C/EBPβ levels in various types of EBV (+) cells after clofoctol treatment. As shown in Figure 2-4, C/EBPβ transcript level was increased in various EBV-positive cell lines, including EBV (+) epithelial cancer cell lines and lymphoma cell lines. The same result was observed in LCLs with both RNA and protein levels.

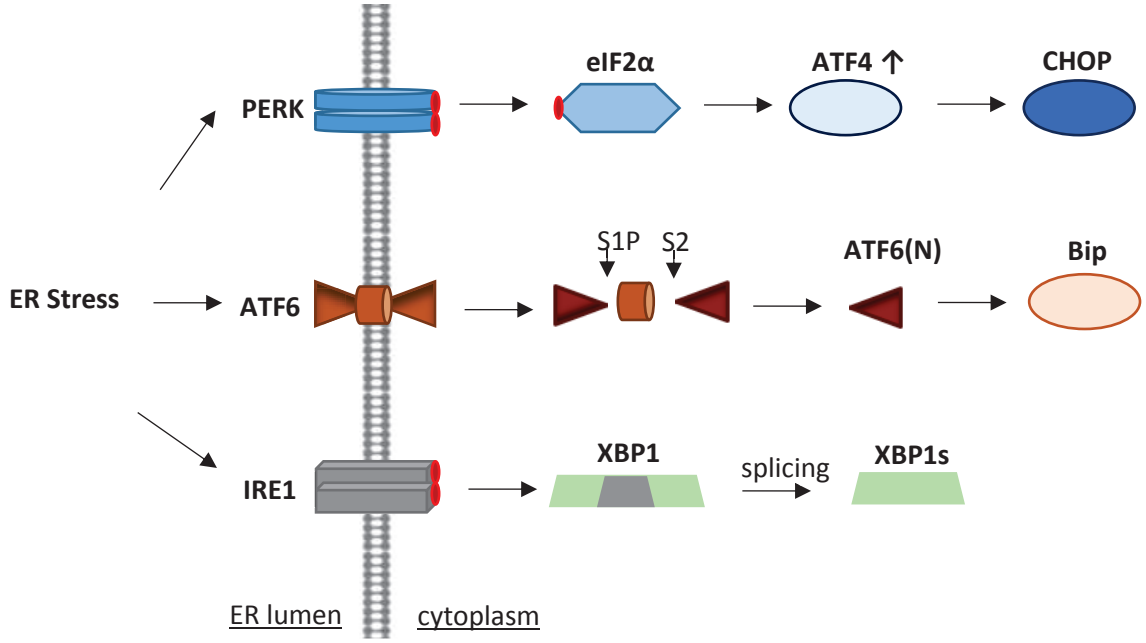


Figure 2-1. Unfolded Protein Response

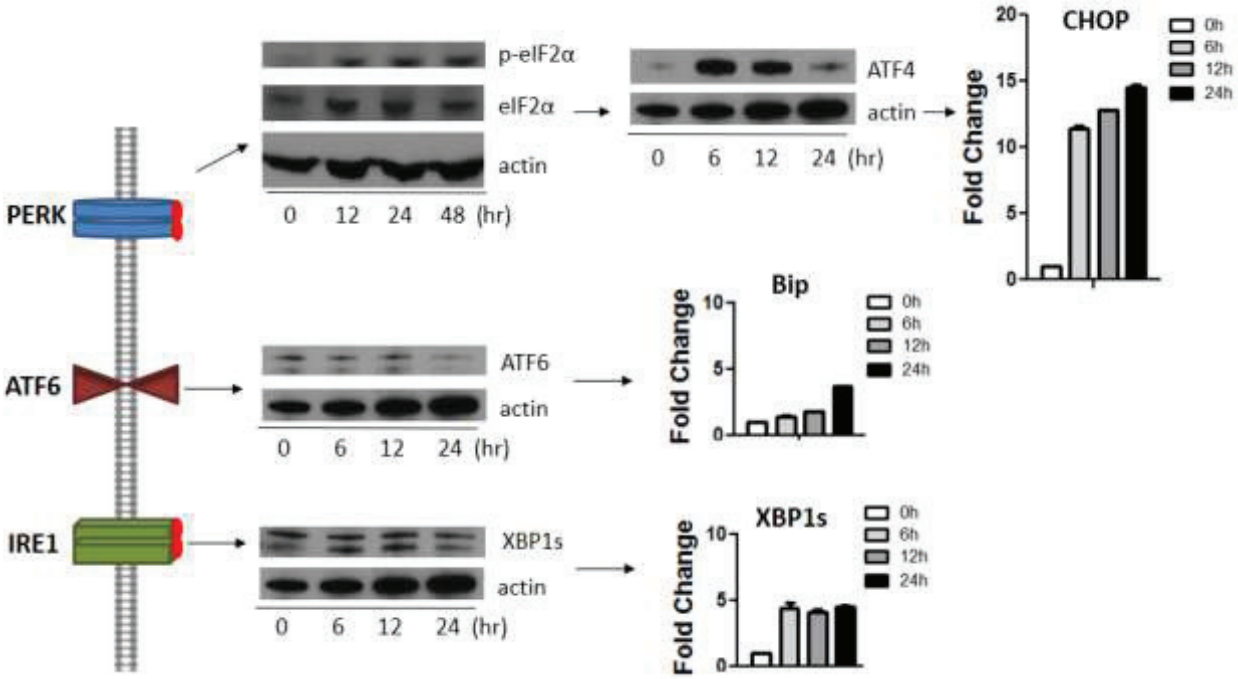


Figure 2-2. Clofoctol activates the UPR in BX1-Akata cells

BX1-Akata cells were treated with 20 uM clofoctol, proteins were extracted at the indicated time points and immunoblots were performed with antibody against eIF2α, p-eIF2α, ATF4, XBP1s, ATF6 and actin. RNAs were isolated at the indicated time points and reverse-transcribed cDNA were used for qPCR with CHOP, XBP1s, Bip and GAPDH primers.

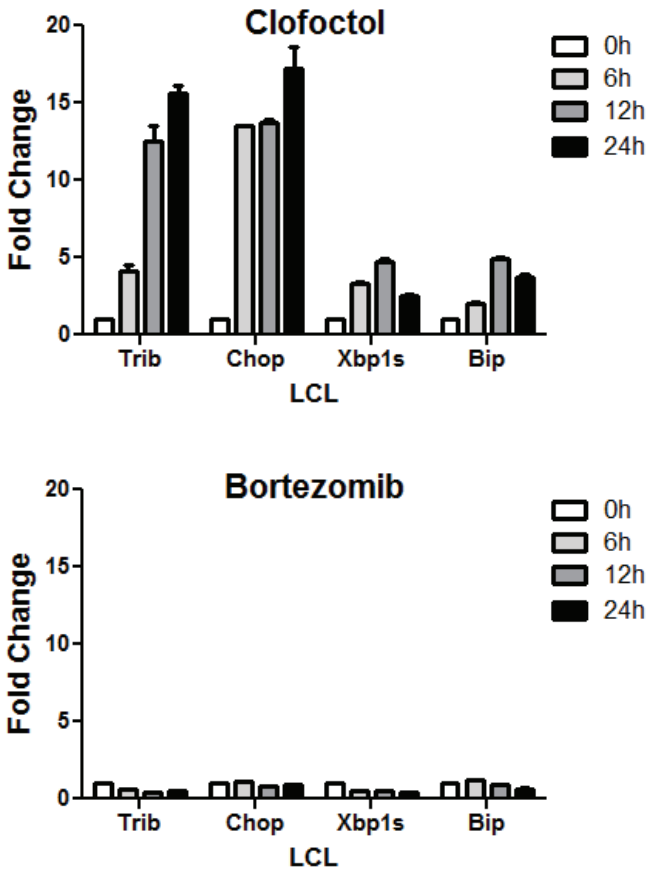
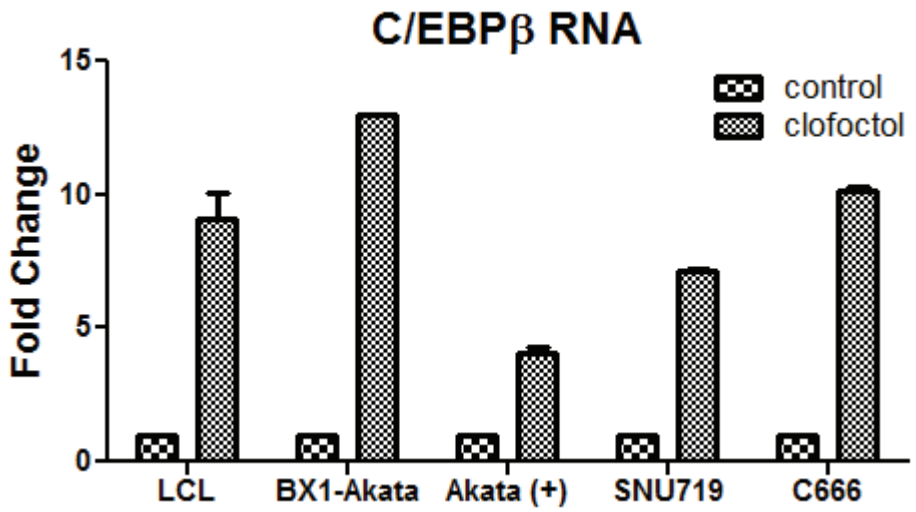


Figure 2-3. Clofoctol activates the UPR in LCL, but bortezomib does not

LCL cells were treated with 20 uM clofoctol or 20 nM bortezomib, RNAs were isolated at the indicated time points and reverse-transcribed cDNA were used for qPCR with Trib, CHOP, XBP1s, Bip and GAPDH primers.

(A)



(B)

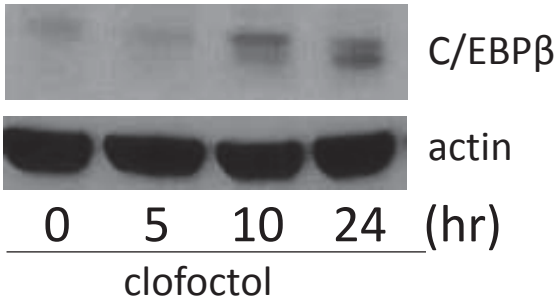


Figure 2-4. Clofoctol increases C/EBPβ expression

LCL, BX1-Akata, Akata EBV (+), SNU719 and C666 cells were treated with 20 uM clofoctol, RNAs were isolated 24 hours later and qRT-PCR was performed to detect C/EBPβ transcript level (A). Protein was extracted at the indicated time points from LCL and immunoblot was performed with antibody against C/EBPβ and actin (B).

DISCUSSION

There are several mechanisms to pharmacologically induce the UPR. Thapsigargin and clotrimazole are two drugs shown to activate the UPR by the disruption of calcium homeostasis in the ER, tunicamycin works through the inhibition of N-linked glycosylation of proteins and bortezomib inhibits the 26S proteasome, thus accumulated mis/unfolded proteins trigger ER stress and the UPR [90, 91]. The molecular mechanism underlying the induction of UPR by clofoctol is unclear. When comparing clofoctol's effect on the UPR with bortezomib in BX1-Akata cells, UPR target gene expression was similarly increased, suggesting their shared pathway events.

However, the level of lytic induction depends on cellular background, and more specifically, the patient sources where the cell line derived from determines the cellular response [72]. For example, TPA, the most widely used agent in labs for EBV lytic induction in majority of EBV (+) cell lines, does not work in LCL. Even within the same type of cell lines, the same lytic inducer treatment brings different cellular outcomes. For example, LCLs derived from different patients show different epigenetic patterns and different levels of EBV copy number per cell, resulting in differential responses to lytic inducers. This was evidenced by the report showing thapsigargin-induced ER stress is sufficient to trigger lytic replication including viral gp350 protein expression and virion release, but this effect was only observed in high-copy (>400 copies/cell) LCLs, not in low-copy (<50 copies) LCLs [56]. It was also reported that LCL are relatively resistant to the UPR, which was attributed to the high EBNA3C expressions [92]. Here, we also confirmed that bortezomib, an UPR inducer well-characterized in various cell lines, did not trigger the UPR in LCL. This could explain the inability of bortezomib to induce

EBV lytic replication in this type of cell line. Interestingly, the UPR in LCL was activated by clofoctol treatment. This suggests that although clofoctol and bortezomib might have shared pathways, the two drugs work through their distinct mechanisms and the clofoctol effect might be less cell type-dependent.

CHAPTER 3: THE PERK PATHWAY AND EBV LYTIC ACTIVATION

SUMMARY

It is well known that the unfolded protein response induces EBV lytic activation [69], and this was further confirmed by several studies showing that chemical agents which induce the UPR can activate EBV lytic infection [60]. Previous investigators showed that upregulated C/EBPβ and XBP1s by the UPR mediates Z promoter expression [37, 60]. However, the exact mechanism of EBV reactivation by the UPR is not clear.

Among three branches of the UPR, the PERK pathway plays as an important regulator of global protein synthesis and cellular fates in response to stress stimuli. PERK leads to eIF2α phosphorylation, which in turn inhibits most translation, but allows some RNAs, including ATF4, to be translated. By using the PERK inhibitor and shRNA knockdown for PERK, we investigated the effect of PERK activity on EBV lytic gene expression induced by clofoctol. We found that knocked-down or inhibited PERK leads to reduced Zta expression. This suggests that Zta protein is being made with PERK activation and eIF2α phosphorylation, indicating that Zta translation overcomes the block introduced with eIF2α phosphorylation. In contrast, viral late gene gp350 which was not induced by clofoctol alone, was induced with clofoctol in combination with the PERK inhibitor. Here, we show the differential effect of PERK inhibition on viral lytic protein expression.

BACKGROUND

ER transmembrane receptor PERK is bound to the BiP chaperone protein and exists in an inactive state under normal physiological conditions [93]. Once PERK senses the accumulated misfolded or unfolded proteins in the ER, it dissociates from the BiP, oligomerizes, autophosphorylates and enters an active state [94]. Activated PERK phosphorylates α subunits of eIF2. eIF2 is composed of three subunits - α, β and γ, and strictly controls translation initiation in eukaryotic cells [95]. First, it binds to guanosine triphosphate (GTP) and initiator-methionyl-tRNA ((Met)-$tRNA_i$) and forms a ternary complex**.** Then, this complex forms a preinitiation complex 43S with the smaller ribosomal subunit 40S and eukaryotic initiation factors, eIF1, eIF1A and eIF3. A heterotetramer eukaryotic initiation factor 4F (eIF4F) brings mRNA into the preinitiation complex 43S, thus enabling the proper AUG initiation codon pairing. The resulting complex is called the 48S preinitiation factor and this process requires hydrolysis of GTP from eIF2α, which is facilitated by the GTPase-activating protein eIF5. Subsequently, the eIF2-GDP complex and all the other eukaryotic initiation factors are released [96] and the 60S subunit of the ribosome binds to the 48S pre-initiation complex to complete the 80S initiation complex formation [97]. In order to generate a new ternary complex, eIF2β needs to replace the GDP to GTP [98] and this process is tightly controlled by the phosphorylation of eIF2α [99]. Under ER stress conditions, activated PERK phosphorylates eIF2α at serine 51 [100] and this stops the 5'cap-dependent global protein translation and allows preferential translation of selected mRNAs such as ATF4, CHOP, and GADD34 [101-103] that have a short upstream open reading frame (uORF) in their 5' untranslated region (5'UTR) [104]. It was also found that ATF4 mRNA bypasses the Cap

recognition and translation starts from the direct recruitment of ribosomes to internal ribosome entry sites (IRES) [105], suggesting that interaction of both uORF and IRES elements within the 5'UTR of the stress gene regulate the cap-independent translation initiation [106].

ATF4 is a basic leucine zipper (bZIP) transcription factor, which belongs to the activating transcription factor/cyclic AMP response element binding protein (ATF/CREB) family [107]. Under stress conditions, preferentially translated ATF4 drives transcriptional upregulation of stress-responsive genes involved in cellular amino acid metabolic processes, mRNA translation, and the unfolded protein response (UPR), such as CHOP and Trib3 [108]. Overall, the regulation of global protein synthesis is the major result of eIF2α phosphorylation and ATF4, which ultimately determines the cellular outcome [109].

As dysregulation of protein synthesis brings various types of pathologic consequences such as diabetes, cancer and neurodegenerative disease, strategies aiming to pharmacologically modulate this pathway have been well developed [110, 111]. For example, GSK2606414 was developed to prevent the PERK autophosphorylation, thus trapping the PERK in an inactive state [112]. Another small-molecule inhibitor, which works downstream of eIF2α phosphorylation, is called integrated stress response inhibitor (ISRIB). It has been shown to attenuate ATF4 synthesis and restore the global translational capacity [113].

As the PERK-ATF4 pathway plays as a major regulatory role in protein synthesis and its activation by clofoctol was confirmed, we sought to investigate the effect of the PERK pathway on clofoctol-induced EBV lytic gene expression.

MATERIALS AND METHODS

Cell Culture. BX1-Akata is an engineered derivative Akata line which carries a recombinant EBV that constitutively expresses a green fluorescent protein (GFP) and was cultured in RPMI 1640, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10% v/v fetal bovine serum (FBS) and 500 μg/mL G418 (geneticin; Life Science Technologies).

Reagents. Clofoctol was a gift from J. Liu (Johns Hopkins University School of Medicine) and purchased from Sigma-Aldrich. PERK inhibitor (GSK2606414) and ISRIB were obtained from MilliporeSigma.

qRT-PCR. RNA was extracted using an RNeasy Mini Kit (QIAGEN) and reverse-transcribed into cDNA by using iScript reverse synthase kit (Bio-Rad). Reverse transcription was performed using a CFX96 real time thermocycler (Bio-Rad). SsoFast Evagreen Supermix (Bio-Rad) with 500 nM primers and cDNA corresponding to 25 ng of the RNA was used for each reaction. cDNA was amplified at 95°C for 30 seconds for 1 cycle and 95°C for 5 seconds and 60°C for 10 seconds, for a total of 40 cycles. GAPDH primers were used as a control for normalization. GAPDH primers used were Forward (5'-TCTTTTGCGTCGCCAGCCGA-3') and GAPDH Reverse (5'-AGTTAAAAGCAGCCCTGGTGACCA-3'). EBV Zta primers used were Forward (5'-ACATCTGCTTCAACAGGAGG-3') and EBV Zta Reverse (5'-AGCAGACATTGGTGTTCCAC-3'). Trib primers used were Forward (5'-CGTGATCTCAAGCTGTGTCG-3') and Trib Reverse (5'-AGCTTCTTCCTCTCACGGTC-3'). The PERK primer set was purchased from Santa Cruz biotechnology.

Immunofluorescence. For staining cells with Cy3, 1.5 x 10^5 cells were spun onto microscope slides by using a Cytospin centrifuge, fixed/permeabilized with ice-cold methanol for 15 minutes, blocked in PBS with 5% Bovine serum albumin (BSA) for 30 minutes, incubated with anti-EBV Zta or gp350 mouse antibody at 1:50 for 1 hour, washed three times for ten minutes each with 5% BSA, 0.1% Tween-20 in PBS and Cy3 goat anti-mouse antibody (Jackson Immunoresearch) was applied to the cells for 1 hour at room temperature. After 3 final washes, the cells were stained with Vectashield mounting media with DAPI (Vector Laboratories). The ZOE Fluorescent cell imager (Bio-Rad) was used for cell fluorescence detection.

shRNA knockdown. shRNA Plasmids consisting of a pool of three to five lentiviral vector plasmids designed to knockdown PERK gene expression were purchased from Santa Cruz biotechnology. Transduction of the lentiviral particles was performed according to the manufacturer's protocol and stable cell lines expressing the shRNA were isolated via selection with puromycin.

RESULTS

ER stress triggers PERK dependent phosphorylation of eukaryotic translation initiation factor 2 alpha (eIF2α) and translation of ATF4 is selectively upregulated by the phosphorylated eIF2α. To investigate whether PERK mediates the effects of clofoctol, we tried to modulate the activity of PERK by either using a PERK inhibitor (GSK2606414) or short hairpin RNA (shRNA) lentiviral vector plasmids to knockdown PERK gene expression. We also used another small molecule inhibitor of the pathway called integrated stress response inhibitor (ISRIB), which reverses the phosphorylated state of eIF2α [113]. RNA level of ATF4 target gene Trib was measured as an indicator of the PERK activity (Fig. 3-1).

As shown in Figure 3-2, clofoctol treatment alone increased Trib and Zta RNA expression as well as GFP expression in BX1-Akata cells and PERK inhibitor treatment resulted in decreases of the expressions induced by clofoctol. To further validate the impact of blocking PERK, BX1-Akata cells were transfected with shRNA targeting PERK and the puromycin selected cells were treated with or without clofoctol. We found clofoctol mediated increased Zta RNA and GFP expressions were inhibited in the presence of shPERK (Fig. 3-3). Blocking the downstream of eIF2α phosphorylation by ISRIB also reduced the increased level of Trib, Zta RNA and GFP expression by clofoctol in BX1-Akata cells (Fig. 3-4). This result suggests that the PERK pathway mediates EBV lytic activation by clofoctol. Activated PERK leads to eIF2α phosphorylation which in turn inhibits most translation, but allows some RNAs including ATF4 to be translated. In our results, Zta protein synthesis was enhanced with PERK activation by clofoctol treatment, suggesting Zta translation overcomes the eIF2α

phosphorylation mediated translational block. Furthermore, we found the PERK pathway differentially regulates viral lytic gene expression at the protein level. Clofoctol treatment alone triggers expression of the immediate early protein Zta. However, Zta expression was reduced by treatment with a PERK inhibitor. In contrast, expression of the viral late lytic protein gp350 was not increased by clofoctol alone but was increased by the combination of clofoctol and PERK inhibitor (Fig. 3-5). This result suggests that clofoctol activates other branches of the UPR and that these also are important in driving EBV lytic gene expression (Fig. 3-6).

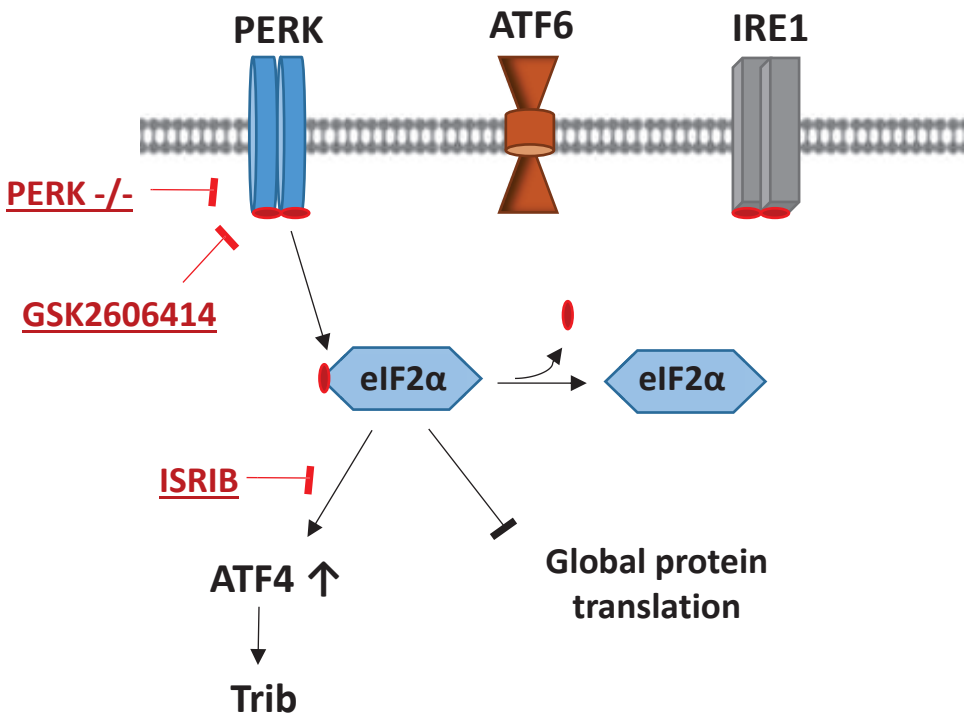


Figure 3-1. Modulation of PERK activity

Treatment of GSK2606414 for inhibiting PERK activity or short hairpin RNA (shRNA) lentiviral vector plasmids to knockdown PERK gene expression. ISRIB targets downstream events of eIF2α phosphorylation.

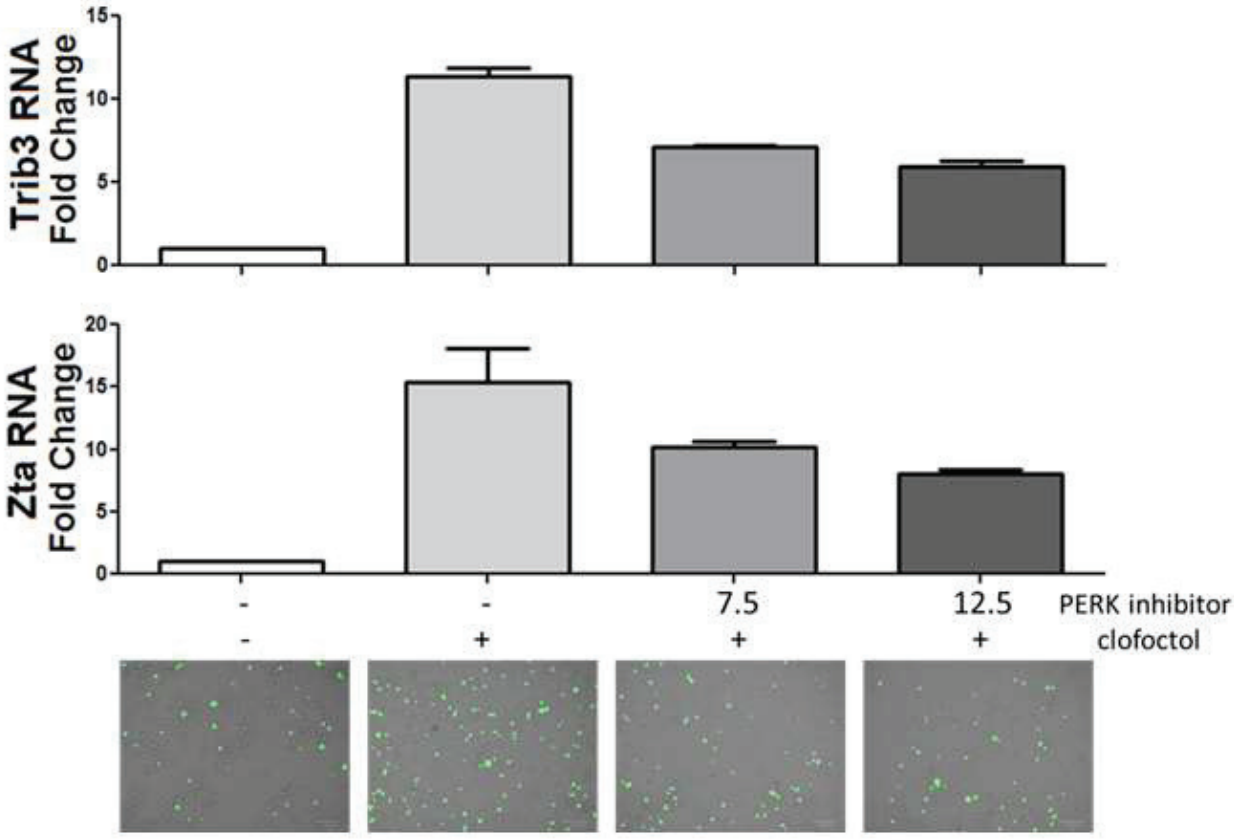


Figure 3-2. An inhibitor of PERK reduces EBV Zta activation by clofoctol

BX1-Akata cells were pre-treated with indicated doses of PERK inhibitor (uM) for 30 minutes, followed by clofoctol or no treatment. 24 hours later, the GFP (+) cells were detected by fluorescence microscopy and isolated RNA was used for quantification of Trib and Zta transcript level.

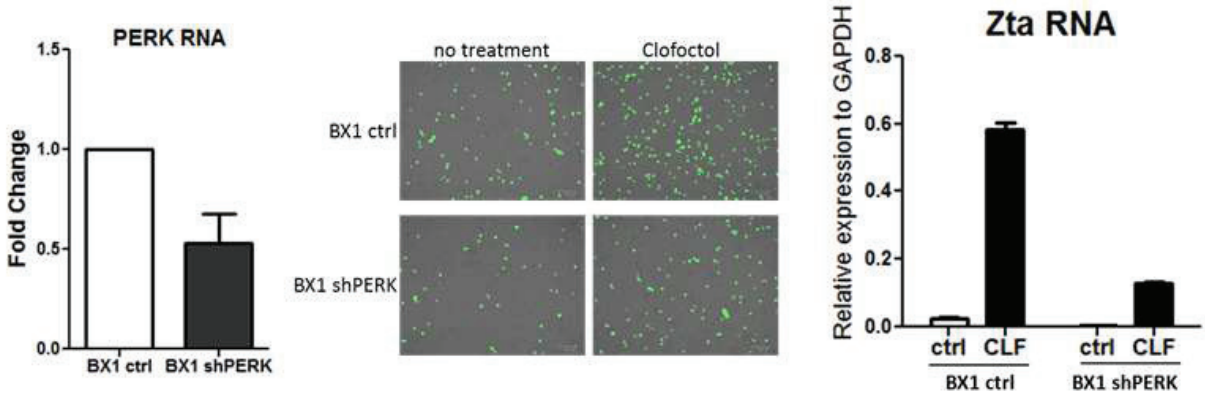


Figure 3-3. shRNA knockdown of PERK reduces EBV Zta activation by clofoctol

BX1-Akata cells were transfected with shRNA lentiviral vector plasmids designed to knockdown PERK gene expression. After the transfection, cells with shRNAs were selected by puromycin. qRT-PCR was performed to confirm the PERK knockdown and the Zta RNA quantification after 24 hours of clofoctol treatment. Fluorescence microscopy was used for detecting the GFP (+) cells after 24 hours of clofoctol treatment.

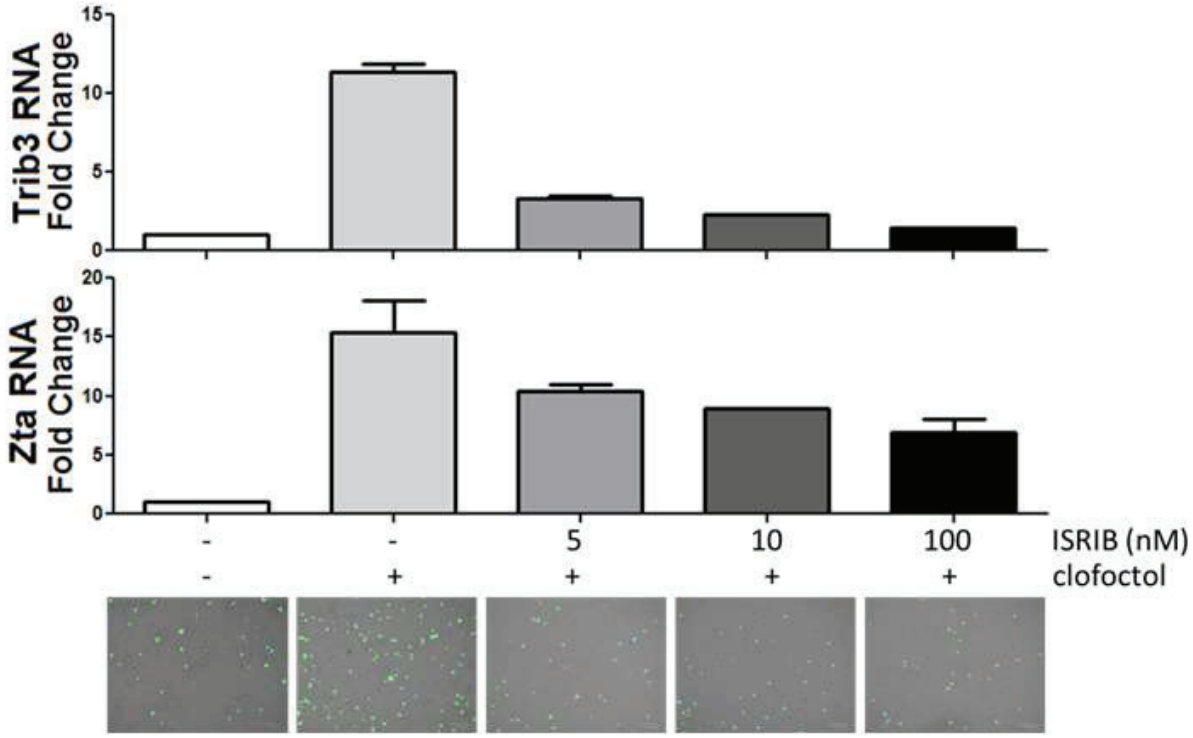


Figure 3-4. ISR inhibitor (ISRIB) reduces EBV Zta activation by clofoctol

BX1-Akata cells were pretreated with ISRIB for 30 minutes and followed by either clofoctol or no treatment. RNA was isolated for Zta and Trib RNA quantification 24 hours after the treatment. Fluorescence microscopy was used for detecting the GFP (+) cells 24 hours after the treatment.

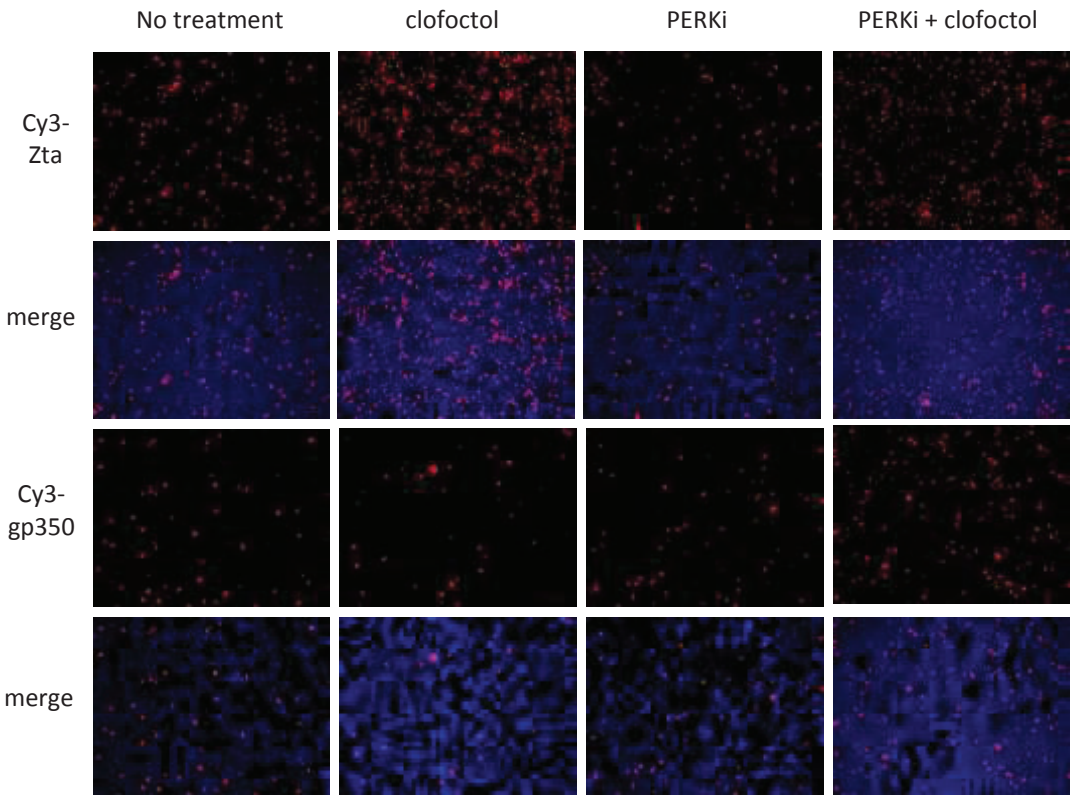


Figure 3-5. Differential effect of PERK inhibition on viral protein expressions

BX1-Akata cells were pretreated with PERK inhibitor (PERKi) 7.5 uM for 30 minutes and followed by clofoctol (20 uM) treatment. 24 hours later, immunofluorescence was performed to detect Zta and gp350 protein expressions. RNA was isolated to measure Trib3 RNA level.

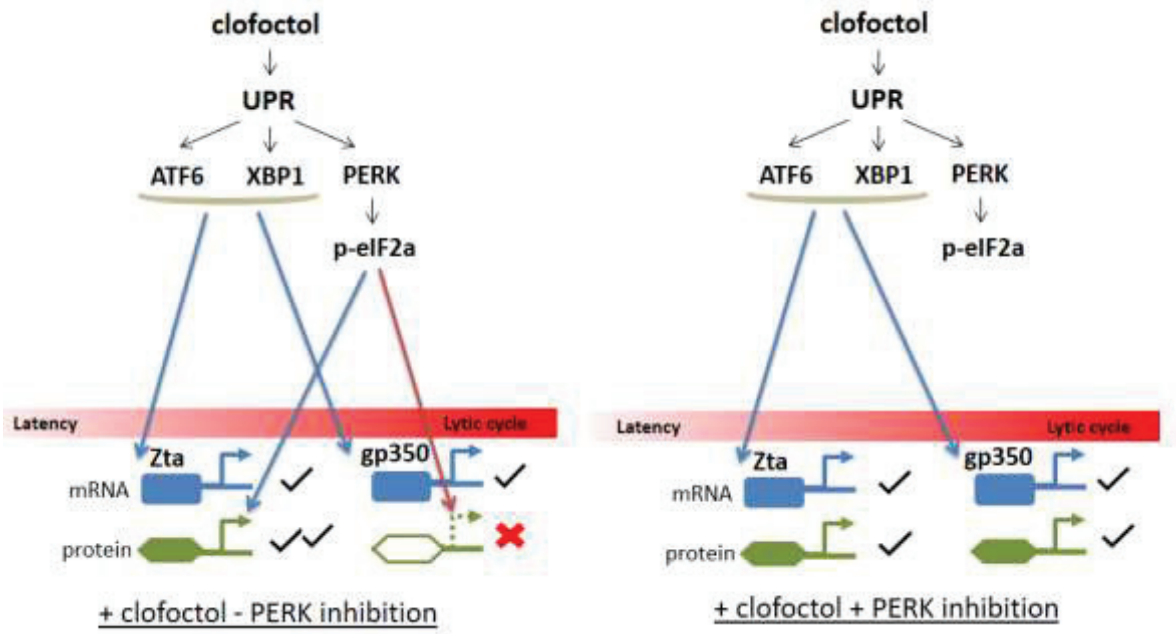


Figure 3-6. Effect of PERK inhibition on viral protein expression

DISCUSSION

Activated PERK prevents ternary complex formation by eIF2α phosphorylation in order to attenuate global protein translation. Our results show that clofoctol treatment activates the PERK pathway, as evidenced by the increase of eIF2α phosphorylation, ATF4 and CHOP expression and this accounts for the inhibited viral gp350 protein expression despite the high mRNA levels seen. However, we saw increased Zta protein expression with clofoctol treatment, indicating that Zta translation overcomes PERK-mediated translation inhibition. We also found that inhibition of PERK resulted in reduced expression of Zta by clofoctol treatment, suggesting that the expression is at least partially mediated by the PERK pathway.

When cells are exposed to environmental stress, it is critical to regulate the rate of translation for adaptation and cell fate decision. There are two broad categories of protein regulation. One is blocking the global protein translation and the other is selective translation of proteins which are essential for the cellular stress response [114]. Under stress conditions, mTOR or eIF2α kinases inhibit the translation initiation by affecting the eIF4E or eIF2 ternary complex formation in the cap-dependent mechanism. Whereas, the selective translation of protein is mediated by sequence signatures present in the 5' untranslated regions (UTR) in the stress genes which enable the translation initiation without the cap recognition from the initiation factors. Several functional elements have been found, and internal ribosome entry site (IRES) and upstream open reading frame (uORF) are the examples. IRES allows direct recruitment of 40S ribosome to the site which is called internal initiation and many reports verified that the selective IRES-mediated protein translation plays important role for determining cellular fate in various

types of pathogenesis [115, 116]. IRES dependent translation was first identified in poliovirus [117] and has since been characterized in other viruses including KSHV [118] and EBV [119]. The presence of IRES within the 5'UTR of the EBNA1 promotes cap-independent translation and enhances EBNA1 protein expression. uORF is another transcript sequences found in 5'UTR of stress genes such as ATF4. Under normal conditions, ribosomes engage only in the uORF, but under stress conditions, ribosomes pass through the uORF and start the translation of the main coding ORF. Interactions of both uORF and IRES elements within the 5'UTR of the stress gene regulate the translation initiation in cap-independent manner [106]. Additionally, another form of eIF2α-independent translation was revealed, which is mediated by eIF5B instead of eIF2 for translation initiation [120]. This process is called eIF5B-dependent translation initiation and the increased level of eIF5B accompanied by the high level of eIF2α phosphorylation is demonstrated in various types of stress conditions [121, 122].

Our findings suggest the possibility that translation initiation of Zta is regulated in a non-classical way of cap-independent or eIF2α-independent manner and further experiments are required to elucidate the precise mechanism of Zta overcoming the translation block.

Another interesting finding we observed with the PERK inhibition assay was the enhanced protein expression of gp350. Clofoctol treatment alone did not elicit gp350 protein expression. Presumably, it was inhibited by the PERK-mediated translation block as it appeared with the PERK inhibitor. The appearance of gp350 with clofoctol and PERK inhibitor treatment suggests that the other UPR pathways are contributing to viral lytic protein expression. Additionally, the level of Zta protein expression with clofoctol

and PERK inhibitor treatment was slightly higher than the basal level, also suggesting stimulation from the other UPR pathways. One such pathway is the IRE1-XBP1 pathway. Under ER stress, the UPR sensor IRE1 homodimerizes, autophosphorylates and catalyzes the splicing of XBP1 mRNA, resulting in a spliced isoform of XBP1 (XBP1s). After being translated, it regulates the transcription of various cellular genes as a transcription factor and its expression is required for cellular secretory process, increase of protein synthesis as well as terminal B cell differentiation [123, 124]. Additionally, it was shown to directly bind to the Z promoter and upregulates Z promoter activation [36, 37]. It is apparent that activated XBP1 as a result of clofoctol treatment might also contribute to viral lytic gene expression.

The products of uORF translation of the stress response genes were predicted to be MHC I peptides active in adaptive immunity [125]. As combinational treatment of clofoctol and PERK inhibitor drive immediate early and late viral protein expression and normal host T cell response effectively reacts to viral lytic antigens, our results suggest possible therapeutic implications that the combinational strategy might increase the targets for immune cell mediated killing.

CHAPTER 4: THE INTEGRATED STRESS RESPONSE AND EBV LYTIC ACTIVATION

SUMMARY

A range of physiological stress conditions were found to induce the integrated stress response (ISR), such as hypoxia, amino acid deprivation and viral infection. There are four different kinases that phosphorylated eIF2α in response to their specific stress stimuli: PERK, HRI, PKR and GCN2. A variety of chemical reagents were discovered to specifically enhance kinase activity. Clofoctol-induced EBV lytic activation mediated by PERK raised the possibility that pharmacological activation of other eIF2α kinases might induce viral activation as well. To further investigate whether activated ISR signaling by different stimuli promotes Zta expression, BX1-Akata cells were treated with four different chemical compounds shown to trigger each kinase's activity. CCT020312 was used for PERK activation, arsenite was used for HRI activation, poly(I:C) was nucleofected into cells for PKR activation and halofuginone was treated for GCN2 activation. Lastly, nelfinavir was treated for enhancing the eIF2α phosphorylation by blocking the dephosphorylation process. The treatments all induced EBV Zta RNA expression and increased viral replication was indirectly indicated by enhanced GFP expression in BX1-Akata cells.

BACKGROUND

PERK phosphorylation of eIF2α has long been recognized as a branch of the UPR, but other kinases associated with distinct stress pathways also lead to phosphorylation of eIF2α. The integrated stress response (ISR) refers to the convergence of these stress signaling pathways on phosphorylation of eIF2α [126]. In addition to PERK, the other kinases that phosphorylate eIF2α are double-stranded RNA-dependent protein kinase (PKR), heme-regulated eIF2α kinase (HRI), and general control non-derepressible 2 kinase (GCN2). These 4 eIF2α kinases responses to their distinct stress stimuli phosphorylate the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α) in order to turn off global protein synthesis and selected genes, which do not require Cap recognition by the eIF4F complex, are therefore being preferentially translated [126].

First, PERK is located in the endoplasmic reticulum (ER) membrane and its activation is derived from ER stress, which is also a part of the unfolded protein response. One of the compounds that selectively activated PERK without triggering a general UPR was discovered and shown to promote eIF2α phosphorylation [127]. HRI in erythroid cells is activated by the absence of heme [128], arsenite-induced oxidative stress [129] and 26S proteasome inhibition [130]. A chemical compound called BTdCPU has been found to specifically target HRI activation [131]. GCN2 is activated in response to amino acid deprivation [132], and halofuginone, a cancer drug in clinical trials, was shown to activate GCN2 and phosphorylates eIF2α [133, 134]. PKR is activated mainly by double-stranded RNA (dsRNA) during viral infection [135]. It was also shown that nucleofection, even in the absence of the delivered nucleic acid, leads to transient eIF2α phosphorylation by GCN2 and PERK [136].

The downstream point of regulation of the ISR signal is the dephosphorylation of eIF2α to restore the protein synthesis [137]. Reversing the phosphorylation of eIF2α is mediated by protein phosphatase 1 (PP1) and there are two cellular cofactors of PP1: GADD34 (PPP1R15A) and CReP (Constitutive Repressor of eIF2α Phosphorylation, PPP1R15B). GADD34 terminates the response by specifically directing PP1 to dephosphorylate eIF2α [137], and CReP maintains eIF2α phosphorylation in a low level [138]. Recently, it was reported that IRE1 branch of the UPR increases eIF2α phosphorylation by degrading the mRNA of CReP, indicating that CReP expression is controlled in an IRE1-dependent manner in order to efficiently regulate protein synthesis [139]. Several clinically used drugs were shown to enhance the ISR by regulating the activity of GADD34 or CReP. For example, the HIV protease inhibitor Nelfinavir was recently shown to activate the ISR by downregulating CReP level and decrease the dephosphorylation activity of PP1, thus enhancing eIF2α phosphorylation [140].

To better understand the impact of ISR on EBV lytic activation, we utilized pharmacological activation of ISR signaling and evaluated the effect on virus in BX1-Akata Burkitt lymphoma cells.

MATERIALS AND METHODS

Cell Culture. BX1-Akata is an engineered derivative Akata line which carries a recombinant EBV that constitutively expresses a green fluorescent protein (GFP) and was cultured in RPMI 1640, 2 mM L-glutamine, 100 IU/mL penicillin, 100 μg/mL streptomycin, 10% v/v fetal bovine serum (FBS) and 500 μg/mL G418 (geneticin; Life Science Technologies).

Reagents. Clofoctol was purchased from Sigma-Aldrich. Nelfinavir, EIF2AK3 activator (CCT020313), Polyinosinic:polycytidylic acid (poly (I:C)) and arsenite were purchased from MilliporeSigma. Halofuginone was purchased from Cayman chemical.

qRT-PCR. RNA was extracted from the RNeasy Mini Kit (QIAGEN) and reverse-transcribed into cDNA by using iScript reverse synthase kit (Bio-Rad). Reverse transcription was performed using a CFX96 real time thermocycler (Bio-Rad). SsoFast Evagreen Supermix (Bio-Rad) with 500 nM primers and cDNA corresponding to 25 ng of the RNA was used for each reaction. cDNA was amplified at 95°C for 30 seconds for 1 cycle and 95°C for 5 seconds and 60°C for 10 seconds, for a total of 40 cycles using a CFX96 real time thermocycler. GAPDH primers were used as a control for normalization. GAPDH primers used were Forward (5'-TCTTTTGCGTCGCCAGCCGA-3') and GAPDH Reverse (5'-AGTTAAAAGCAGCCCTGGTGACCA-3'). EBV Zta primers used were Forward (5'-ACATCTGCTTCAACAGGAGG-3') and EBV Zta Reverse (5'-AGCAGACATTGGTGTTCCAC-3'). EBV Bmrf1 primers used were Forward (5'-CTAGCCGTCCTGTCCAAGTGC-3') and EBV Bmrf1 Reverse (5'-AGCCAAACGCTCCTTGCCCA-3'). EBV gp350 primers used were Forward (5'-

GTCAGTACACCATCCAGAGCC-3') and EBV gp350 Reverse (5'-TTGGTAGACAGCCTTCGTATG-3').

Nucleofection. Poly(I:C) nucleofection was performed by using an Amaxa nucleofector device according to the manufacturer's protocol from the Amaxa Cell Line Nucleofector Kit .

RESULTS

There are four eIF2α kinases (PERK, HRI, PKR and GCN2) which sense distinct environmental and physiological stresses. Phosphorylation of eIF2α triggers the integrated stress response (ISR). We treated BX1-Akata cells with four compounds each of which stimulates a different eIF2α kinase and monitored lytic activation (Fig. 4-1). CCT020312 was used to activate PERK, arsenite was used to activate HRI, poly(I:C) was nucleofected into cells to activate PKR, and halofuginone was used to activate GCN2. 24 hours after treatment, we performed qRT-PCR to measure Zta gene expression, and GFP expression was detected by fluorescence microscopy. We found that treatment by all four eIF2α kinase stimulators led to increases in Zta RNA expression and GFP expression in BX1-Akata cells.

A previous report showed that nelfinavir induces the ISR by inhibiting the dephosphorylation of eIF2α (Fig. 4-3) [140]. We confirmed the nelfinavir-mediated ISR activation which was indicated by upregulation of Trib gene expression and also found increased viral lytic gene expression after nelfinavir treatment.

Together these data show that activation of eIF2α phosphorylation by various ISR stimuli leads to viral lytic gene expression.

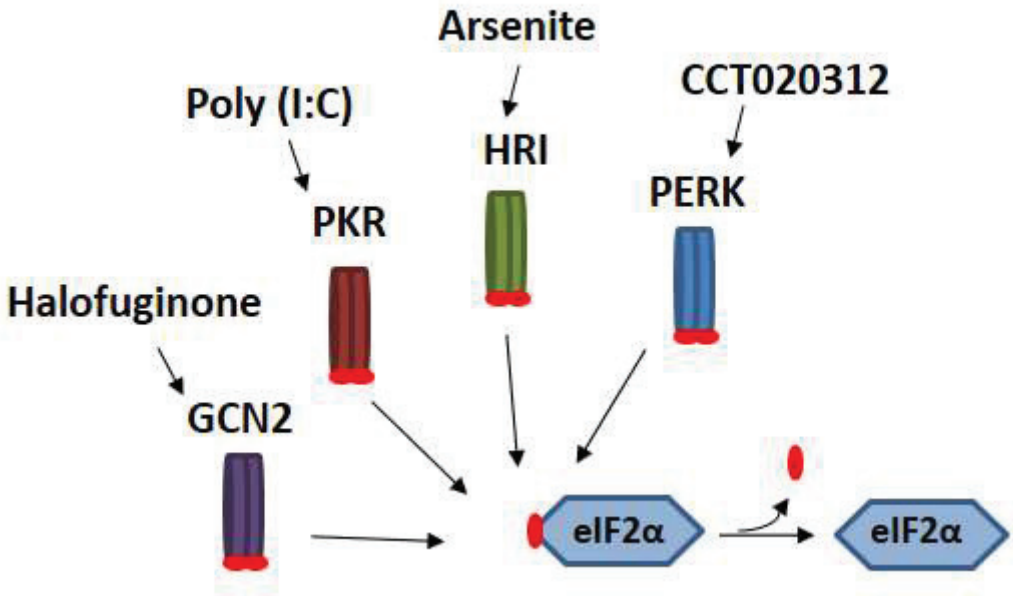


Figure 4-1. Integrated stress response stimulation

CCT020313 (7 uM), poly (I:C) (10 ug/ml), arsenite (50 uM) and halofuginone (70 nM) were used for ISR stimulation.

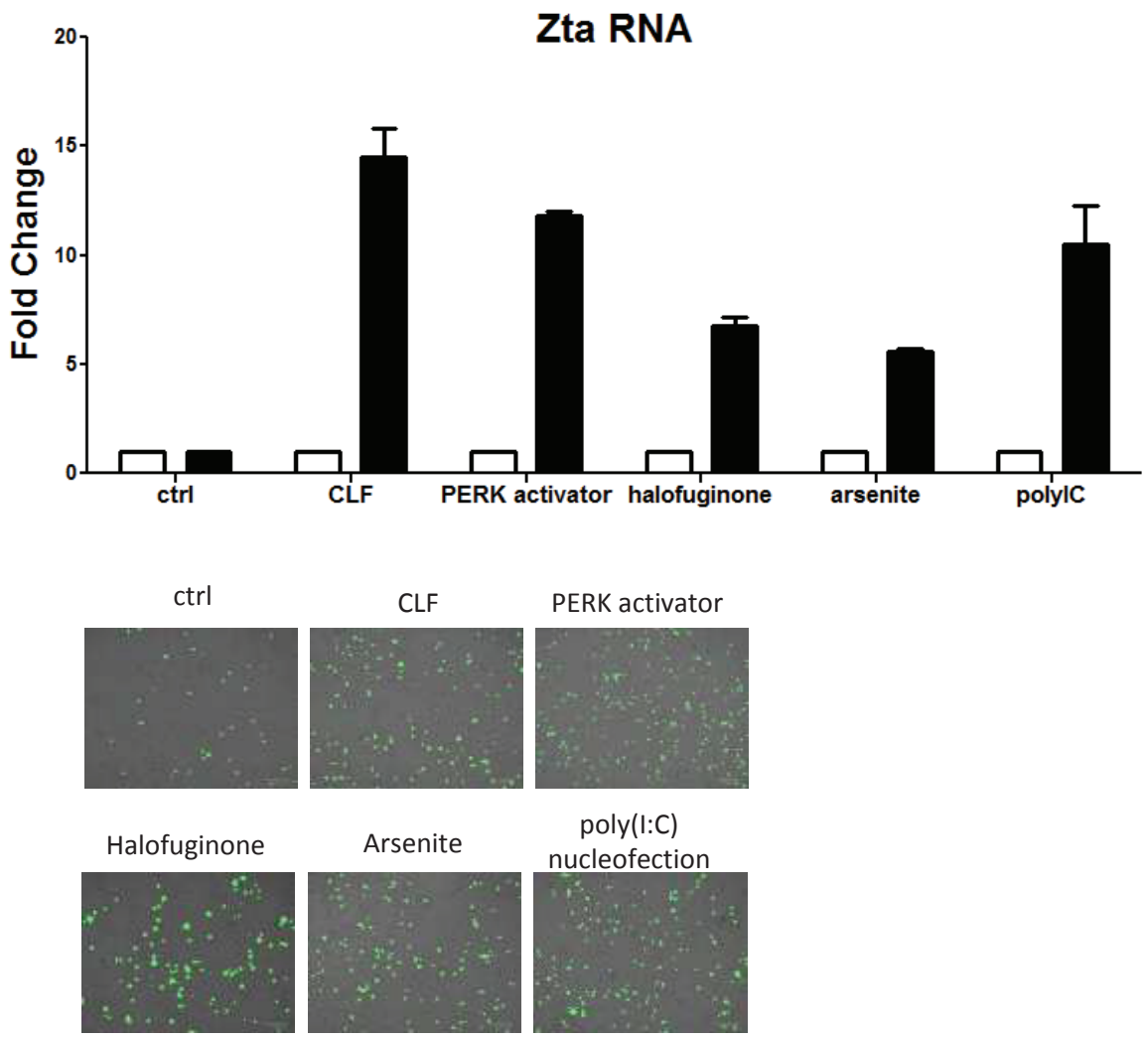
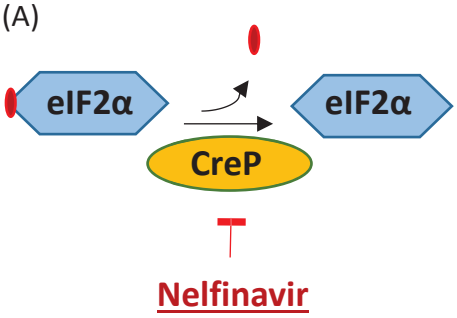


Figure 4-2. ISR stimuli activate Zta gene expression and increase GFP expression in BX1-Akata cells

BX1-Akata cells were treated with clofoctol (20 uM), PERK activator (CCT020313, 7 uM), arsenite (50 uM) and halofuginone (70 nM), and nucleofected with poly(I:C) (10 ug/ml) for the ISR stimulation. 24 hours later, RNA was isolated for quantification of Zta transcript level and GFP positive cells were detected by fluorescence microscopy.

(A)



(B)

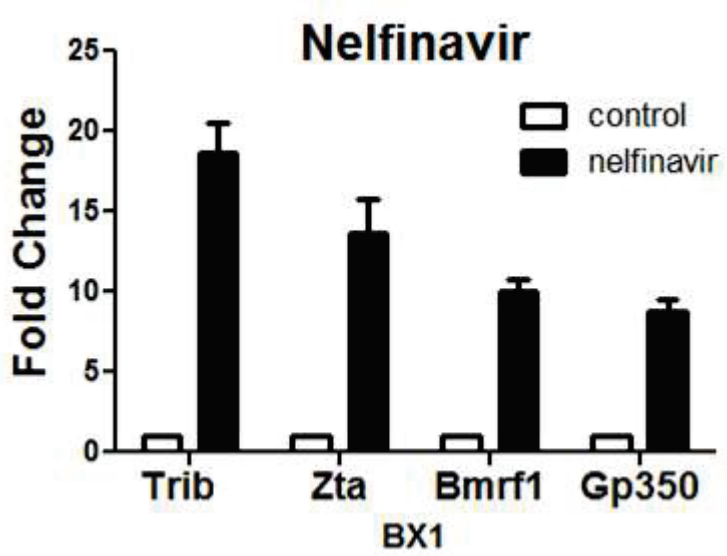


Figure 4-3. Nelfinavir activates the ISR and EBV lytic gene expression

A. Nelfinavir inhibits the constitutive eIF2α dephosphorylation by downregulation of the phophatase cofactor CReP (Constitutive Repressor of eIF2α Phosphorylation; also known as PPP1R15B).

B. BX1-Akata cells were treated with nelfinavir (20 uM) for 24 hours and isolated RNA was used for quantification of Trib, Zta, Bmrf1 and gp350 transcript level.

DISCUSSION

Here we show several reagents that specifically activate eIF2α phosphorylation also induce the EBV lytic gene expression thus confirming that ISR pathways lead to EBV lytic activation. In combination with evidence that clofoctol leads to eIF2α phosphorylation, this provides evidence that clofoctol activation of EBV lytic infection can be accounted for, at least in part, by activation of the ISR.

Arsenite exposure leads to the dose-dependent and tissue-dependent apoptosis and has been used for the treatment of certain types of cancers such as acute promyelocytic leukemia and multiple myeloma [141]. One of the most immediate cellular responses to arsenite exposure is inhibition of protein synthesis by phosphorylation of the eIF2α through HRI and such a drastic reduction of translation plays a cytoprotective role [129].

Halofuginone (Tempostatin), a synthetic quinazolinone alkaloid derivative, was shown to have anti-angiogenic, anti-metastatic, and anti-proliferative effects in preclinical studies [142] and was studied in a phase II study in patients with AIDS related Kaposi sarcoma (KS)(AMC 036) [143].

Nelfinavir is an HIV aspartyl protease inhibitor. *In vitro* and *in vivo* studies showed anti-cancer effects related to induction of apoptosis, ER stress and autophagy even in the absence of HIV infection [144]. It was recently shown that nelfinavir affects translation in two different ways [145]. Nelfinavir enhances the phosphorylation of eIF2α to inhibit the translation initiation [140] and activates eukaryotic elongation factor 2 kinases (eEF2K) leading to the phosphorylation of the elongation factor eEF2, thereby blocking translation elongation [146]. It was suggested that the cytotoxic effects of

nelfinavir reflect alterations in translation that are similar to those resulting from inhibition of mTORC1 by rapamycin [146].

Drug repositioning is considered as a low-risk and low-cost strategy that has been widely used to identify new clinical opportunities for old drugs. As some of drugs we tested are already approved for use in humans, it would be advantageous to further investigate its possible implication for EBV associated cancer treatment.

Although pharmacological agents were used in this study to elicit the ISR, various environmental and physiological conditions were well-known to trigger the pathway. For example, GCN2 becomes active in amino acid deprived condition [132]. HRI becomes active by heme deficiency for the production of hemoglobin and can also be activated by other stresses including oxidative stress, heat shock, and osmotic stress [147]. Further investigations may provide a broader insight to understand relevant physiologic stimuli that trigger EBV reactivation *in vivo*.

CONCLUSIONS

In this study, we found a drug that activates EBV in diverse latently infected cell lines. Clofoctol has been widely used in Europe as an antibiotic and is well-tolerated. The effects of the drug on viral gene regulation have not been previously reported. Its effects on mammalian cell stress pathways was revealed following a drug screen to identify compounds that were toxic to prostate cancer cell lines [64]. The previous report suggested that clofoctol works on the unfolded protein response (UPR) and inhibits prostate cancer cell growth. We also confirmed the activation of the UPR pathway by clofoctol, which results in EBV lytic gene expression in various types of EBV-harboring cancer cell lines. Clofoctol treatment drives the transcription of EBV lytic genes and translation of immediate early lytic protein Zta, but does not induce the translation of late lytic proteins or production of infectious virions.

Upon the stimulation of a stress signal, cells stop producing peptides from conventional translation in order to conserve resources and survive under stressful conditions. Stress response kinases phosphorylate eIF2α, leading to downregulation of eIF2-dependent translation from an AUG start codon. Under ER stress, one UPR sensor, PERK, becomes active and induces phosphorylation of eIF2α, thus stopping global translation. In this report, we found that the EBV immediate early lytic protein Zta was being made in the presence of phosphorylated eIF2α, indicating that the translation of Zta escapes the global translational inhibition.

A recent study supported the idea of T cell recognition of the peptide translated from uORF after stress-induced eIF2α phosphorylation [125]. To be detected by T cells, the intracellular protein must be degraded, loaded onto an MHC 1 molecule and

presented on the cell surface. Then, the peptide can be recognized by a T cell with a T-cell receptor specific for that antigen. This process is highly sensitive, as T cells are able to detect even a few copies of the peptide–MHC I complex. From our results, it is possible to predict that the EBV Zta protein which escaped from translational downregulation might ultimately be presented into the T cell recognition mechanism.

Furthermore, our results showed that the expression of the viral late lytic protein gp350 appeared with co-treatment of clofoctol and a PERK inhibitor. This result suggests that clofoctol activates other branches of the UPR that are also driving EBV lytic gene expression and attenuation of the translational block by the PERK inhibitor led to gp350 expression.

The EBV genome encodes more than 80 open reading frames and most of them elicit immunologic responses. We hypothesize that combination treatment with clofoctol and a PERK inhibitor will drive expression of most lytic genes, enhancing functional T cell responses.

Although viral activation from the UPR has been well studied, our study broadens insights into stress stimuli that affect viral activation. Not only ER stress, but also other environmental stress conditions which drive the ISR could induce viral activation, and this may help our understanding of viral activation *in vivo*.

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Project: Post-translational regulations of tumor suppressors in breast cancer development.

PUBLICATIONS

Lee J, Kosowicz JG, Liu JO, Hayward SD, Ambinder RF. An antibiotic triggers cell stress responses and Epstein Barr virus lytic activation, *Manuscript in progress*

Lee J, Kosowicz JG, Ambinder RF. Unveiling Kaposi sarcoma viral antigens. Oncotarget (2017). 8(31): 50325–50326 doi: 10.18632/oncotarget.19106

Kosowicz JG*, Lee J*, Peiffer B, Guo Z, Chen J, Liao G, Hayward SD, Liu JO, Ambinder RF. Drug modulators of B cell signaling pathways and Epstein-Barr virus lytic activation. Journal of Virology (2017) 91(16). pii: e00747-17 doi: 10.1128/JVI.00747-17

Kosowicz JG, Lee J, Ambinder RF. Cancer: Seeing the ebb of a tumour virus. Nature Biomedical Engineering, News and Views (2017) doi:10.1038/s41551-017-0059

PRESENTATIONS

16th International Conference on Malignancies in HIV/AIDS (Bethesda, USA)

Title: Epstein–Barr virus activation by nelfinavir is linked to the JNK pathway and autophagy.

17th International Symposium on EBV and associated diseases (Zurich, Switzerland)

Title: Rapamycin does not block B cell receptor-mediated EBV lytic induction.

15th International Conference on Malignancies in AIDS and Other Acquired Immunodeficiencies (Bethesda, USA)

Title: Recently approved kinase inhibitors for the treatment of B-cell malignancies block B-cell–receptor mediated EBV lytic activation.

SCHOLARSHIPS

Graduate Teaching Assistant Scholarship (2013) - Course: Pathology for Graduate Students – Basic Mechanisms

Margaret Lee fellowship (2012 – 2013) - Department of Pathology, Johns Hopkins University School of Medicine

Student President Scholarship (2010) - Department of Chemistry, Yonsei University

Yonsei College Scholarship (2008 – 2011)
